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(71) Applicant *(for all designated States except US)*: M & E
BIOTECH A/S [DK/DK]; Kogle Allé 6, DK–2970 Hør-
sholm (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: STEINAA, Lucilla
[DK/DK]; Viktoriagade 28, 3. th., DK–1655 København
V (DK). MOURITSEN, Søren [DK/DK]; Lindevangsvej
24, DK–3460 Birkerød (DK). NIELSEN, Klaus, Gregorius
[DK/DK]; Lykkesborg Allé 15, DK–2860 Søborg (DK).
HAANING, Jesper [DK/DK]; Biskop Svanes Vej 2E,
DK–3460 Birkerød (DK). LEACH, Dana [US/DK]; Ved
Store Dyrehave 44, DK–3400 Hillerød (DK). DALUM,
Iben [DK/DK]; Olgasvej 13, DK–2970 Hørsholm (DK).
GAUTAM, Anand [GB/DK]; Ved Store Dyrehave 22,
st.tv., DK–3400 Hillerød (DK). BIRK, Peter [DK/DK];
Rudolph Berghsgade 5, DK–2100 København ø (DK).

KARLSSON, Gunilla [SE/DK]; Collinsgade 6A, 3. tv.,
DK–2100 København ø (DK).

(74) Agent: PLOUGMANN, VINGTOFT & PARTNERS A/S;
Sankt Annae Plads 11, P.O. Box 3007, DK–1021 Copen-
hagen K (DK).

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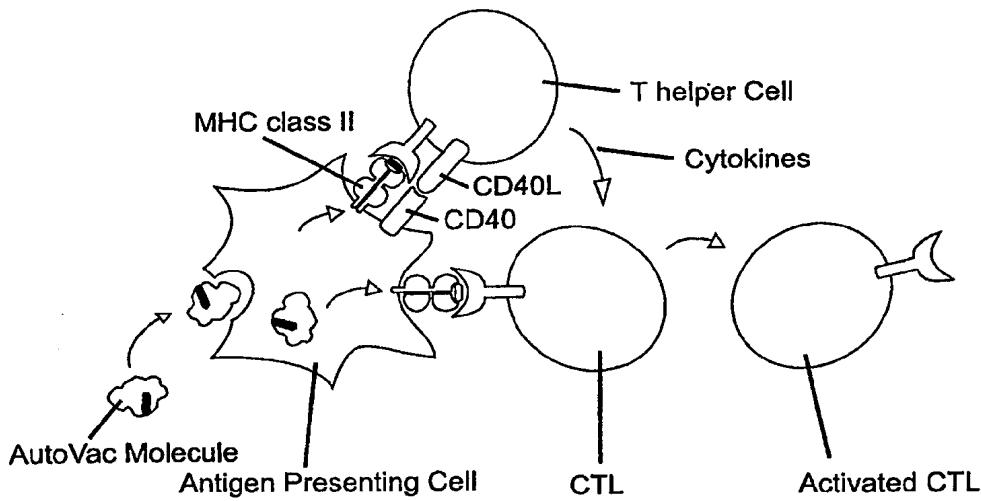
(57) Abstract

A method is disclosed for inducing cell–mediated immunity against cellular antigens. More specifically, the invention provides for
a method for inducing cytotoxic T–lymphocyte immunity against weak antigens, notably self–proteins. The method entails that antigen
presenting cells are induced to present at least one CTL epitope of the weak antigen and at the same time presenting at least one foreign
T–helper lymphocyte epitope. In a preferred embodiment, the antigen is a cancer specific antigen, e.g. PSM, Her2, or FGF8b. The
method can be exercised by using traditional polypeptide vaccination, but also by using live attenuated vaccines or nucleic acid vaccination.
The invention furthermore provides immunogenic analogues of PSM, Her2 and FGF8b, as well as nucleic acid molecules encoding these
analogues. Also vectors and transformed cells are disclosed. The invention also provides for a method for identification of immunogenic
analogues of weak or non–immunogenic antigens.

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NOVEL METHODS FOR THERAPEUTIC VACCINATION

FIELD OF THE INVENTION

The present invention relates to novel methods for combatting diseases, such as cancers, which are characterized by the
5   presence of cell-associated gene expression products which are non-immunogenic or poorly immunogenic. In particular, the present invention relates to methods for inducing an immune response conducted by cytotoxic T-lymphocytes (CTLs), whereby cells carrying epitopes from the gene expression products are
10 attacked and killed by the CTLs. The invention also relates to a method of preparing immunogenic, modified polypeptide antigens which are derived from weakly immunogenic antigens.

The invention further relates to a series of applications of Applicants' AutoVac technology (which is the subject of WO
15 95/05849) within the field of therapeutic vaccination against cancer.

BACKGROUND OF THE INVENTION

The idea of vaccinating against cancer has been around for more than hundred years and has enjoyed recurrent bursts of
20 activity, particularly since the turn of this century.

However, during the past 10 years the understanding of the fundamental molecular mechanisms of the immune response has improved considerably. Among the most important milestones achieved during this period has been the discovery of the
25 still growing list of cytokines and growth factors, the understanding of the mechanisms of interaction between T and B cells as well as the establishment of the cellular antigen processing pathways including the role and structure of the MHC class I and II molecules in antigen presentation. Impor-

tant discoveries with regard to cancer immunology - although
still not fully understood - were also the elucidation of the
mechanisms underlying induction of immunological tolerance in
a host. All this research has led to a huge amount of efforts
5 in order to develop new treatments for human cancer.

Depending on how tumour immunity is acquired by the patient,
immunotherapy regimens can be categorised as either passive or
active. In passive immunotherapy regimens the patient passive-
ly receives immune components such as cytokines, antibodies,
10 cytotoxic T-cells, or lymphocyte activated killer (LAK) cells.
In contrast, active specific immunotherapy protocols encompass
actively inducing tumour immunity by vaccination with the
tumour cell or its antigenic components. This latter form of
treatment is preferred because the immunity is prolonged.

15 Passive and active cancer vaccines have focussed on inducing
either humoral or cellular immune responses. For active vac-
cines it is well established that induction of CD4 positive T
helper cells is necessary in order to secondarily induce
either antibodies or cytotoxic CD8 positive T cells.

20 *Passive vaccination with antibodies*

Since the discovery of the monoclonal antibody technology in
the mid-seventies, a large number of therapeutic monoclonal
antibodies directed against tumour specific or tumour associ-
ated antigens has been developed. Monoclonal antibody therapy,
25 however, gives rise to several serious problems:

-       Injection of these foreign substances induces an immune
        response in the patient towards the injected antibodies,
        which may lead to less efficient treatment as well as to
        serious allergic side-effects in the patients.

-       Monoclonal antibodies usually must be administered in
        large amounts. This is a problem, since the production
        costs of monoclonal antibodies are huge.

-       Monoclonal antibodies must be administered via the
5   parenteral route and due to the relatively large amounts
        needed, the patients frequently must be hospitalised
        during the treatment.

-       Injections of monoclonal antibodies must be repeated at
        rather short intervals (weeks) in order to maintain
10   therapeutic effect.

-       Monoclonal antibodies are usually not able to activate
        secondary effector systems of the immune system such as
        complement, NK-cells or macrophage killing of tumour
        cells.

15 The latter disadvantage is of particular importance in cancer
   therapy and may be an important reason why monoclonal antibody
   therapy of cancer in several cases has not been particularly
   successful. The so-called humanised monoclonal antibodies now
   used by many companies are less immunogenic, but unfortunately
20 they are even less capable of activating the secondary immune
   effector systems. Furthermore, examples of secondary out-
   growth of tumours lacking the original tumour antigen have
   been observed, since these antibodies do not induce "innocent
   bystander" effects on tumour cells not carrying the tumour
25 antigen.

   The poor effector capability of the monoclonal antibodies has
   led to the development of monoclonal antibodies chemically
   conjugated to different toxins and radioisotopes. Pharmacia
   Upjohn AB has e.g. developed a conjugate between a monoclonal
30 tumour specific antibody and the *Staphylococcus aureus* toxin A

with the purpose of activating T cells in the tumour. Medarex Inc. has developed bispecific monoclonal antibodies containing a tumour specific Fab fragment as well as an Fc-receptor specific antibody fragment with the purpose of activating

5 macrophage killing of tumour cells. Both constructs are more effective than the monoclonal antibody alone, but they are also more expensive and immunogenic. Antibodies conjugated to radioisotopes are also expensive as well as immunogenic and other general toxic side-effects are observed.

10 The appearance of the monoclonal antibody technology was a major step forward which enabled the production of well-defined, high-affinity binding molecules. However, being monoclonal these antibodies only react with a single type of epitope on a tumour antigen. This is the major reason why they

15 usually are not able to activate the complement system or binding to the Fc-receptors of NK-cells and macrophages. These very powerful effector systems usually require the co-localisation of multiple Fc antibody fragments protruding from the antigen.

20 Other researchers have therefore attempted to use two monoclonal antibodies in combination and this has led to an improved effect. It therefore seems very reasonable instead to attack tumour cells with highly specific polyclonal antibodies directed against a tumour specific, or against (over-expressed)

25 tumour associated antigens or growth factor receptors. Such antibodies would be fully capable of activating the secondary effector systems mentioned above. Furthermore, it is likely that the local inflammatory reaction induced by these effector systems could lead to secondary effects on "innocent by-

30 stander" cells not expressing the tumour antigen in question as well as to activation of tumour specific TIL´s (tumour infiltrating lymphocytes) in the tumour tissue. Such effects

have been observed by Medarex Inc. using their bi-specific
monoclonal antibody conjugates.

Since the discovery of the monoclonal antibody technology the
potential use of polyclonal antibodies for cancer therapy has
5 not been explored very much (except for the antigens described
below). One major reason is that well-defined tumour specific
or tumour associated surface antigens only have been charac-
terised within the recent years, but - more importantly - many
of these have turned out to be self-antigens and therefore
10 non-immunogenic. Accordingly, xenogenic polyclonal antibodies
would necessarily have been used to study the effects. How-
ever, such antibodies induce a vigorous immune response to-
wards the injected foreign polyclonal antibodies which rapidly
eliminate the therapeutic effects.

15 *Active vaccination to induce antibodies*

Recent attempts to induce therapeutic polyclonal autoantibo-
dies in cancer patients by active vaccination have been suc-
cessful. Vaccines against membrane bound carbohydrate self-
antigens (such as the O-linked aberrantly expressed Tn and
20 sTn-antigens and the ganglioside liposaccharides GM2 and GD3)
have been developed. These small carbohydrate structures are,
however, very poor antigens so conjugates of these molecules
with carrier molecules such as keyhole limpet haemocyanin
(KLH) or sheep mucins (containing Tn- and sTn) must be used.
25 In melanoma patients the induction of anti-GM2 antibodies were
associated with a prolonged disease-free interval and overall
survival after a minimum follow-up of fifty-one months. Also
randomised phase II studies have been conducted on breast
cancer patients using a conjugate of sTn and KLH in the DETOX-
30 B adjuvant (BIOMIRA Inc.) showing that sTn immune patients had
a significantly longer median survival compared to controls.
Another example of the active induction of polyclonal antibo-

dies in cancer is the use of idiotype specific vaccination
against B-cell lymphomas, which - although it has been promis-
ing - is limited to this cancer type only.

Finally, the US company Aphton Inc. has developed active
5   conjugate vaccines against gonadotropin releasing hormone
(GnRH) and gastrin. It has been demonstrated, that this vac-
cine is capable of controlling the biological activity of
these hormones, which also can function as autocrine growth
factors for certain tumour cells. Successful phase II clinical
10 trials have been conducted on gastrointestinal cancer patients
and phase III clinical trials are underway.

Cytotoxic T-cells

It has been clearly demonstrated by several groups that tumour
specific cytotoxic T cells (CTL´s) are present in many tu-
15 mours. These CTL's are termed tumour infiltrating lymphocytes
(TIL's). However, these cells are somehow rendered non-respon-
sive or anergic by several different possible mechanisms
including secretion of immunosuppressive cytokines by the
tumour cells, lack of co-stimulatory signals, down regulation
20 of MHC class I molecules etc.

There has been many attempts to isolate the tumour specific
HLA class I bound peptides recognised by TILs, and in some
cases it has also been successful (e.g. peptides from the
melanoma associated antigens). Such peptides have been used to
25 induce a tumour specific immune response in the host, but the
practical use of tumour specific peptides in vaccines is
restricted to a limited segment of the population due to the
narrow HLA class I binding specificity of the peptides. Fur-
thermore, it is usually relatively difficult to evoke a CTL
30 response *in vivo* using synthetic peptides due to the low

biological half-life of these substances as well as the diffi-
culties with exogenous priming of MHC class I molecules.

Many other approaches have been attempted in order to evoke a
tumour specific CTL response including the use of cytokines
5 (e.g. IL-2, IFN-γ, IL-6, IL-4, IL-10 or GM-CSF) or co-
stimulatory molecules (B7) either in soluble form or expressed
by the transfected tumour cell. Furthermore, immunisations
with allogenic or autologous whole cells, or of tumour anti-
gens prepared in specialised adjuvants designed to present the
10 antigen via the MHC class I antigen presentation route, or
tumour antigens expressed in e.g. vaccinia vectors etc. have
been used with varying success. Still the general belief among
tumour immunologists is therefore that one of the best ways to
eliminate tumours would be to induce a strong specific anti-
15 tumour CTL response.

Apart from the fact that these treatments usually are very
expensive and difficult to reproduce, it has also turned out
to be difficult to obtain a good immune response towards the
tumour since many of the tumour associated antigens are true
20 self-proteins to which most T cells appear to be tolerant.
Therefore, it seems necessary to induce a controlled cellular
autoimmune condition in the patient.

OBJECT OF THE INVENTION

It is an object of the present invention to provide improved
25 methods and agents for inducing immune responses in host
organisms against undesirable antigens, e.g. tumour antigens.
It is a further object to provide a method for preparing
polypeptide analogues of such undesirable antigens, analogues
which are capable of inducing an effective immune response
30 against the undesired antigen.

SUMMARY OF THE INVENTION

Presentation of antigens has dogmatically been thought of as
two discrete pathways, a class II exogenous and a class I
endogenous pathway.

5 Briefly, a foreign protein from outside the cell or from the
cell membrane is taken up by the APC as an endosome which
fuses with an intracellular compartment which contains
proteolytic enzymes and MHC class II molecules. Some of the
produced peptides bind to class II, which then are transloca-
10 ted to the cell membrane.

The class I endogenous pathway is characterised by the predo-
minant presentation of cytosolic proteins. This is believed to
occur by proteasome mediated cleavage followed by transpor-
tation of the peptides into the endoplasmic reticulum (ER) via
15 TAP molecules located in the membrane of the ER. In ER the
peptides bind to class I followed by transportation to the
plasma membrane.

However, these 2 pathways are not fully distinct. For example
it is known that dendritic cells and to some extend macro-
20 phages are capable of endocytosing (pinocytosing) extracellu-
lar proteins and subsequently present them in the context of
MHC class I. It has also previously been demonstrated that
using specialised administration routes, e.g. by coupling to
iron oxide beads, exogenous antigens are capable of entering
25 the Class I pathway (Rock, 1996). This mechanism seems cen-
tral, because of the importance of a concomitant expression of
both class I and class II on the same APC to elicit a three
cell type cluster. This three cell type cluster of interaction
has been proposed by Mitchison (1987) and later by other
30 authors. They showed the importance of concomitant presenta-

tion of class I and class II epitopes on the same APC. Accor-
ding to the recently described mechanism for CTL activation
(cf. Lanzavecchia, 1998, Nature **393**: 413, Matzinger, 1999,
Nature Med. **5**: 616, Ridge *et al.*, 1998, Nature **393**: 474,
5   Bennett *et al.*, 1998, Nature **393**: 478, Schoenberger *et al.*,
1998, Nature **393**: 480, Ossendrop *et al.*, 1998, J. Exp. Med
187: 693, and Mackey *et al.*, 1998, J. Immunol **161**: 2094),
professional APCs presenting antigen on MHC class II are
recognized by T helper cells. This results in an activation of
10 the APC (mediated by interaction by CD40L on the T helper cell
and CD40 on the APC). This enables the APC to directly stimu-
late CTLs which are thereby activated. Cf. also Fig. 2.

It has previously been demonstrated that insertion of a
foreign MHC class II restricted T helper cell epitope into a
15 self-antigen results in the provision of an antigen capable of
inducing strong cross-reactive antibody responses directed
against the non-modified self-antigen (cf. applicant's WO
95/05849). It was shown that the autoantibody induction is
caused by specific T cell help induced by the inserted foreign
20 epitope.

However, we have come to the conclusion that modified self-
antigens - with the aid of appropriate adjuvants - ought to be
capable of also inducing strong CTL responses against MHC
class I restricted self-epitopes and hence the technology
25 described in WO 95/05849 can be adapted to also provide vacci-
nation against intracellular and other cell-associated anti-
gens which have epitopes presented in the context of MHC Class
I.

The autovaccine technology described in WO 95/05849 has the
30 effect that specific T cell help is provided to self-reactive
B cells when a modified self-antigen is administered for
uptake into the MHC class II antigen processing pathway (cf.

10

Fig. 1, and Dalum I *et al.*, 1996, J. Immunol. **157**: 4796-4804
as well as Dalum I *et al.*, 1999, Nature Biotechnol. **17**: 666-
669). It was shown that potentially self-reactive B-lympho-
cytes recognizing self-proteins are physiologically present in
5   normal individuals. However, in order for these B-lymphocytes
to be induced to actually produce antibodies reactive with the
relevant self-proteins, assistance is needed from cytokine
producing T-helper lymphocytes (T_H-cells or T_H-lymphocytes).
Normally this help is not provided because T-lymphocytes in
10   general do not recognize T-cell epitopes derived from self-
proteins when presented by antigen presenting cells (APCs).
However, by providing an element of "foreignness" in a self-
protein (*i.e.* by introducing an immunologically significant
modification), T-cells recognizing the foreign element are
15   activated upon recognizing the foreign epitope on an APC (such
as, initially, a mononuclear cell). Polyclonal B-lymphocytes
(which present T-cell epitopes) capable of recognising
self-epitopes on the modified self-protein also internalise
the antigen and subsequently presents the foreign T-cell
20   epitope(s) thereof, and the activated T-lymphocytes subse-
quently provide cytokine help to these self-reactive polyclo-
nal B-lymphocytes. Since the antibodies produced by these
polyclonal B-lymphocytes are reactive with different epitopes
on the modified polypeptide, including those which are also
25   present in the native polypeptide, an antibody cross-reactive
with the non-modified self-protein is induced. In conclusion,
the T-lymphocytes can be led to act as if the population of
polyclonal B-lymphocytes have recognised an entirely foreign
antigen, whereas in fact only the inserted epitope(s) is/are
30   foreign to the host. In this way, antibodies capable of
cross-reacting with non-modified self-antigens are induced.

As mentioned above, CTL's also require specific T cell help,
although the mechanism for this is still not clear.

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We have based the present invention on our novel theory that
the self-proteins containing foreign MHC class II epitopes,
following exogenous uptake, can gain access into the MHC class
I antigen processing pathway of e.g. macrophages and dendritic
5 cells. In this way a strong CTL response against subdominant
epitopes in the self-protein could be induced. Alternatively,
genes encoding modified tumour antigens could be administrated
as nucleic acid vaccines eventually also leading to MHC class
II as well as MHC class I mediated immune responses.

10 Tumour cells are very poor antigen presenting cells due to
insufficient MHC class I expression, lack of co-stimulatory
molecules or secretion of immunosuppressive cytokines etc.
Using the autovaccine constructs and vaccination protocol
mentioned above the modified tumour antigen could be presented
15 by MHC class I as well as by MHC class II molecules on profes-
sional antigen presenting cells. Co-presentation of subdomi-
nant self-epitopes on MHC class I and immunodominant foreign
epitopes on MHC class II molecules would mediate a direct
cytokine help from activated MHC class II restricted T-helper
20 cells to MHC class I restricted CTLs (Fig. 2). This will in
our opinion lead to a specific break of the T cell autotole-
rance towards the tumour antigen and this is exactly what is
desired in cancer immunotherapy.

In conclusion, a vaccine constructed using the technology
25 outlined above will induce a humoral autoantibody response
with secondary activation of complement and antibody dependent
cellular cytotoxicity (ADCC) activity. It is also expected
that it will induce a cytotoxic T cell response directed
against e.g. a tumour specific membrane antigen.

30 Hence, in the broadest and most general scope, the present
invention relates to a method for inducing an immune response
against a polypeptide antigen in an animal, including a human

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being, said polypeptide antigen being weakly immunogenic or
non-immunogenic in the animal, the method comprising effecting
simultaneous presentation by antigen presenting cells (APCs)
from the animal's immune system of an immunogenically effec-
5   tive amount of

1)   at least one CTL epitope derived from the polypeptide
      antigen and/or at least one B-cell epitope derived from
      the cell-associated polypeptide antigen, and
2)   at least one first T helper cell epitope (T_H epitope)
10   which is foreign to the animal.

In a more specific variant of the inventive method, the inven-
tion relates to a method for down-regulating a cell-associated
polypeptide antigen in an animal, including a human being,
said polypeptide antigen being weakly immunogenic or non-
15 immunogenic in the animal, by inducing a specific cytotoxic T-
lymphocyte (CTL) response against cells carrying the cell-
associated polypeptide antigen on their surface or harbouring
the cell-associated polypeptide antigen in their intracellular
compartment, the method comprising effecting, in the animal,
20 simultaneous presentation by a suitable antigen presenting
cell (APC) of

1)   at least one CTL epitope derived from the cell-associated
      polypeptide antigen, and
2)   at least one first T-helper lymphocyte (T_H) epitope which
25   is foreign to the animal.

Also, the novel strategy for preparing an immunogenic agent is
part of the invention. This novel strategy encompasses the
selection and production of analogues of weak cell-associated
antigens, where the preservation of a substantial fraction of
30 known and predicted CTL epitopes is aimed at while at the same
time introducing at least one foreign T_H epitope.

Furthermore, the invention relates to certain specific immuno-
genic constructs based on known tumour-associated antigens as
well as to compositions containing these constructs.

Finally, the invention relates to nucleic acid fragments,
5 vectors, transformed cells and other tools useful in molecular
biological methods for the production of the analogues of the
tumour-associated antigens.

LEGENDS TO THE FIGURE

Fig. 1: The traditional AutoVac concept. A: Tolerodominant
10 self-epitopes presented on MHC class II on an antigen presen-
ting cell (APC) are ignored due to depletion in the T helper
cell (Th) repertoire (T helper cell indicated with dotted
lines). Inserted foreign immunodominant T cell epitopes pre-
sented on MHC class II activate T helper cells and B cells (B)
15 specific for native parts of the self-protein presenting
foreign immunodominant T cell epitopes on MHC class II are
activated by the cytokine help provided by the T helper cell.

Fig. 2: The AutoVac concept for inducing a CTL response.
Inserted foreign immunodominant T cell epitopes presented on
20 MHC class II activate T helper cells. CTL's recognising
subdominant self-epitopes presented on MHC class I are acti-
vated by the adjacent activated T helper cell.

Fig. 3: A schematic representation of the Her2 polypeptide
with indications of epitopic regions and N-glycosylation
25 sites. The 4 extracellular domains, the transmembrane (TM)
domain and the 2 intracellular domains are represented with
indications of sites with varying degrees of homology and
sites containing putative/determined CTL epitopes.

Fig. 4: A schematic representation of the human PSM polypep-
tide with indications of insertion regions for the P2 and P30
epitopes.

Fig. 5: The FGF genes and proteins. A: Exon-intron structure
5   of the human and mouse FGF8 genes. Below is illustrated the
eight different splice forms (from Gemel 1996). B: Amino acid
sequence of the different FGF8 isoforms. The polypeptide
stretches unique to FGF8b, FGF8f, and FGF8e are indicated by
bold and italic or underlined typefaces. FGF8a is the shortest
10   variant containing none of these highlighted sequences. The
signal peptide is expected to be cleaved C-terminally to
Ala22. The two cysteine residues found in mature FGF8(all
isoforms) are indicated by thick underlining. The two poten-
tial N-glycosylation sites of FGF8b are indicated by Ñ. Num-
15   bering is according to FGF8b.

Fig. 6: Illustrations of the four different variants of FGF8b
designed for autovaccination. Upper panel: Theoretical models
of the insertion-points of the epitopes using the FGF2 crystal
structure as template. Lower panel: Amino acid sequences of
20   the wild type FGF8b (WT) and the four variants F30N, F2I,
F30I, and F2C. The signal peptide is marked with single under-
lining. The inserted peptides are marked with double underli-
ning. The N-terminal sequence (MetAla) of all variants is due
to generation of a Kozak-sequence (Kozak 1991) for better
25   translation in eukaryotic systems.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the following a number of terms used in the present
specification and claims will be defined and explained in

15

detail in order to clarify the metes and bounds of the inven-
tion.

A "cell-associated polypeptide antigen" is in the present
specification and claims intended to denote a polypeptide
5 which is confined to a cell which is somehow related to a
pathological process. Furthermore, the cell presents CTL
epitopes of the polypeptide antigen bound to MHC Class I
molecules on its surface. Cell-associated polypeptide antigens
can therefore be truly intracellular antigens (and thereby
10 unreachable for a humoral immune response) or antigens bound
to the surface of the cells. The cell-associated antigen can
be the product of the cell's own gene expression, of a
intracellular parasite, of a virus, or of another cell. In the
latter case the polypeptide antigen is subsequently associated
15 with the cell which is involved in the pathological process.

The terms "T-lymphocyte" and "T-cell" will be used
interchangeably for lymphocytes of thymic origin which are
responsible for various cell mediated immune responses as well
as for effector functions such as helper activity in the
20 humoral immune response. Likewise, the terms "B-lymphocyte"
and "B-cell" will be used interchangeably for antibody-produ-
cing lymphocytes.

An "antigen presenting cell" (APC) is a cell which presents
epitopes to T-cells. Typical antigen-presenting cells are
25 macrophages, dendritic cells and other phagocytizing and
pinocytizing cells. It should be noted that B-cells also
functions as APCs by presenting T_H epitopes bound to MCH class
II molecules to T_H cells but when generally using the term APC
in the present specification and claims it is intended to
30 refer to the above-mentioned phagocytizing and pinocytizing
cells.

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"Helper T-lymphocytes" or "T$_H$ cells" denotes CD4 positive T-cells which provide help to B-cells and cytotoxic T-cells via the recognition of T$_H$ epitopes bound to MHC Class II molecules on antigen presenting cells.

5 The term "cytotoxic T-lymphocyte" (CTL) will be used for CD8 positive T-cells which require the assistance of T$_H$ cells in order to become activated.

A "specific" immune response is in the present context intended to denote a polyclonal immune response directed predomi-
10 nantly against a molecule or a group of quasi-identical molecules or, alternatively, against cells which present CTL epitopes of the molecule or the group of quasi-identical molecules.

A "weak or non-immunogenic polypeptide antigen" is herein
15 intended to denote polypeptides having the amino acid sequence of the weak cell-associated protein antigens derived from the animal in question (e.g. a human), but also polypeptides having the amino acid sequence identical to analogues of such proteins isolated from other species are embraced by the term.
20 Also forms of the polypeptides having differing glycosylation patterns because of their production in heterologous systems (e.g. yeasts or other non-mammalian eukaryotic expression systems or even prokaryotic systems) are included within the boundaries of the term. It should, however, be noted that when
25 using the term, it is intended that the polypeptide in question is normally non-immunogenic or only weakly immunogenic in its natural localisation in the animal to be treated.

The term "polypeptide" is in the present context intended to mean both short peptides of from 2 to 10 amino acid residues,
30 oligopeptides of from 11 to 100 amino acid residues, and polypeptides of more than 100 amino acid residues. Further-

more, the term is also intended to include proteins, *i.e.*
functional biomolecules comprising at least one polypeptide;
when comprising at least two polypeptides, these may form
complexes, be covalently linked, or may be non-covalently
5   linked. The polypeptide(s) in a protein can be glycosylated
and/or lipidated and/or comprise prosthetic groups.

The term "subsequence" means any consecutive stretch of at
least 3 amino acids or, when relevant, of at least 3 nucleo-
tides, derived directly from a naturally occurring amino acid
10   sequence or nucleic acid sequence, respectively.

The term "animal" is in the present context in general inten-
ded to denote an animal species (preferably mammalian), such
as *Homo sapiens*, *Canis domesticus*, etc. and not just one
single animal. However, the term also denotes a population of
15   such an animal species, since it is important that the indi-
viduals immunized according to the method of the invention all
harbour substantially the same weak, cell-associated polypep-
tide antigen allowing for immunization of the animals with the
same immunogen(s). If, for instance, genetic variants of
20   polypeptides exist in different human populations it may be
necessary to use different immunogens in these different
populations in order to be able to break the autotolerance
towards the weak, cell-associated polypeptide antigen in each
population.

25   By the term "down-regulation a cell-associated polypeptide
antigen" is herein meant reduction in the living organism of
the amount and/or activity of the antigen in question. The
down-regulation can be obtained by means of several mecha-
nisms: Of these, simple interference with the active site in
30   the antigen by antibody binding is the most simple. However,
it is also within the scope of the present invention that the
antibody binding results in removal of the polypeptide by

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scavenger cells (such as macrophages and other phagocytizing cells), and even more important, that cells carrying or harbouring the antigen are killed by CTLs in the animal.

The expression "effecting simultaneous presentation by a
5 suitable APC" is intended to denote that the animal's immune system is subjected to an immunogenic challenge in a controlled manner which results in the simultaneous presentation by APCs of the epitopes in question. As will appear from the disclosure below, such challenge of the immune system can be
10 effected in a number of ways of which the most important are vaccination with polypeptide containing "pharmaccines" (i.e. a vaccine which is administered to treat or ameliorate ongoing disease) or nucleic acid "pharmaccine" vaccination. The important result to achieve is that immune competent cells in the
15 animal are confronted with APCs displaying the relevant epitopes in an immunologically effective manner.

The term "immunogenically effective amount" has its usual meaning in the art, i.e. an amount of an immunogen which is capable of inducing an immune response which significantly
20 engages pathogenic agents which share immunological features with the immunogen.

When using the expression that the weak cell-associated polypeptide antigens have been subjected to a "modification" is herein meant a chemical modification of the polypeptide
25 which constitutes the backbone of the polypeptide in question. Such a modification can e.g. be derivatization (e.g. alkylation) of certain amino acid residues in the amino acid sequence, but as will be appreciated from the disclosure below, the preferred modifications comprise changes of the primary struc-
30 ture of the amino acid sequence.

When discussing "tolerance" and "autotolerance" is understood
that since the polypeptides which are the targets of the
present inventive method are self-proteins in the population
to be vaccinated or proteins which do not result in induction
5   of an effective immune response, normal individuals in the
population do not mount an immune response against the
polypeptide. It cannot be excluded, though, that occasional
individuals in an animal population might be able to produce
antibodies against the native polypeptide antigen, e.g. as
10 part of a autoimmune disorder. At any rate, an animal will
normally only be autotolerant towards its own polypeptide
antigen, but it cannot be excluded that analogues derived from
other animal species or from a population having a different
phenotype would also be tolerated by said animal.

15 A "foreign T-cell epitope" is a peptide which is able to bind
to an MHC molecule and stimulates T-cells in an animal spe-
cies. Preferred foreign epitopes are "promiscuous" epitopes,
i.e. epitopes which binds to a substantial fraction of MHC
class II molecules in an animal species or population. Only a
20 very limited number of such promiscuous T-cell epitopes are
known, and they will be discussed in detail below. It should
be noted that in order for the immunogens which are used
according to the present invention to be effective in as large
a fraction of an animal population as possible, it may be
25 necessary to 1) insert several foreign T-cell epitopes in the
same analogue or 2) prepare several analogues wherein each
analogue has a different promiscuous epitope inserted. It
should be noted that the concept of foreign T-cell epitopes
also encompasses use of cryptic T-cell epitopes, *i.e.* epitopes
30 which are derived from a self-protein and which only exerts
immunogenic behaviour when existing in isolated form without
being part of the self-protein in question.

A "foreign T helper lymphocyte epitope" (a foreign T_H epitope) is a foreign T cell epitope which binds an MHC Class Class II molecule and can be presented on the surface of an antigen presenting cell (APC) bound to the MHC Class II molecule.

5   A "CTL" epitope is a peptide which is able to bind to an MHC class I molecule.

A "functional part" of a (bio)molecule is in the present context intended to mean the part of the molecule which is responsible for at least one of the biochemical or physiolo-
10 gical effects exerted by the molecule. It is well-known in the art that many enzymes and other effector molecules have an active site which is responsible for the effects exerted by the molecule in question. Other parts of the molecule may serve a stabilizing or solubility enhancing purpose and can
15 therefore be left out if these purposes are not of relevance in the context of a certain embodiment of the present inven-tion. For instance it is possible to use certain cytokines as a modifying moiety in the analogue (cf. the detailed discus-sion below), and in such a case, the issue of stability may be
20 irrelevant since the coupling to the analogue provides the stability necessary.

The term "adjuvant" has its usual meaning in the art of vac-cine technology, i.e. a substance or a composition of matter which is 1) not in itself capable of mounting a specific
25 immune response against the immunogen of the vaccine, but which is 2) nevertheless capable of enhancing the immune response against the immunogen. Or, in other words, vaccina-tion with the adjuvant alone does not provide an immune re-sponse against the immunogen, vaccination with the immunogen
30 may or may not give rise to an immune response against the immunogen, but the combined vaccination with immunogen and

adjuvant induces an immune response against the immunogen
which is stronger than that induced by the immunogen alone.

"Targeting" of a molecule is in the present context intended
to denote the situation where a molecule upon introduction in
5 the animal will appear preferentially in certain tissue(s) or
will be preferentially associated with certain cells or cell
types. The effect can be accomplished in a number of ways
including formulation of the molecule in composition facili-
tating targeting or by introduction in the molecule of groups
10 which facilitates targeting. These issues will be discussed in
detail below.

"Stimulation of the immune system" means that a substance or
composition of matter exhibits a general, non-specific
immunostimulatory effect. A number of adjuvants and putative
15 adjuvants (such as certain cytokines) share the ability to
stimulate the immune system. The result of using an
immunostimulating agent is an increased "alertness" of the
immune system meaning that simultaneous or subsequent immuni-
zation with an immunogen induces a significantly more effec-
20 tive immune response compared to isolated use of the immunogen

Preferred embodiments

In order to induce a CTL response against a cell which pre-
sents epitopes derived from the polypeptide antigen on its
surface, it is normally necessary that at least one CTL epi-
25 tope, when presented, is associated with an MHC Class I mole-
cule on the surface of the APC. Furthermore it is preferred
that the at least one first foreign T_H epitope, when presented,
is associated with an MHC Class II molecule on the surface of
the APC.

Preferred APCs presenting the epitopes are dendritic cells and
macrophages, but any pino- or phagocytizing APC which is
capable of simultaneously presenting 1) CTL epitopes bound to
MHC class I molecules and 2) T_H epitopes bound to MHC class II
5   molecules, is a preferred APC according to the invention.

According to the invention, the cell-associated polypeptide
antigen is preferably selected from a tumour-associated anti-
gens and other self-proteins which are related to pathological
processes but also viral antigens and antigens derived from an
10   intracellular parasite or bacterium will. It is well-known in
the art that such pathogen-associated antigens are often
relatively poor immunogens (e.g. antigens from mycobacteria
such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*,
but also from protozoans such as *Plasmodium spp.*). It is
15   believed that the method of the invention, apart from render-
ing possible the production of antibody and CTL responses
against true self-protein antigens, is capable of enhancing
the often insufficient immune response mounted by the organism
against such intracellular antigens.

20   Normally, it will be advantageous to confront the immune
system with a large fraction of the amino acid sequence of the
polypeptide antigen which is the vaccine target. Hence, in a
preferred embodiment, presentation by the APC of the CTL
epitope and the first foreign T_H epitope is effected by pre-
25   senting the animal's immune system with at least one first
analogue of the cell-associated polypeptide antigen, said
first analogue comprising a variation of the amino acid se-
quence of the cell-associated polypeptide antigen, said varia-
tion containing at least the CTL epitope and the first foreign
30   T_H epitope. This is in contrast to e.g. a DNA vaccination
strategy where the CTL and T_H epitopes are expressed by the
same cell but as parts of separate polypeptides; such a DNA
vaccination strategy is also an embodiment of the invention,

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but it is believed that having the two epitopes as part of the
same polypeptide will normally enhance the immune response
and, at any rate, the provision of only one expression product
will be necessary.

5   In order to maximize the chances of mounting an effective
immune response, it is preferred that the above-mentioned
first analogue contains a substantial fraction of known and
predicted CTL epitopes of the cell-associated polypeptide
antigen, i.e. a fraction of the known and predicted CTL epi-
10   topes which binds a sufficient fractions of MHC Class I mole-
cules in a population. For instance, it is preferred that the
substantial fraction of known and predicted CTL epitopes in
the amino acid sequence of the analogue are recognized by at
least 50% of the MHC-I haplotypes recognizing all known and
15   predicted CTL epitopes in the cell-associated polypeptide
antigen, but higher percentages are preferred, such as at
least 60, at least 70, at least 80, and at least 90%. Espe-
cially preferred is the use of analogues which preserves
substantially all known CTL epitopes of the cell-associated
20   polypeptide antigen are present in the analogue, i.e. close to
100% of the known CTL epitopes. Accordingly, it is also espe-
cially preferred that substantially all predicted CTL epitopes
of the cell-associated polypeptide antigen are present in the
at least first analogue.

25   Methods for predicting the presence of CTL epitopes are well-
known in the art, cf. e.g. Rothbard et al. EMBO J. 7:93-100
(1988).

As will be apparent from the present specification and claims
it is expected that the inventive method described herein will
30   render possible the effective induction of CTL responses
against cell-associated polypeptide antigens.

24

In cases where the cell-associated polypeptide antigen is truly intracellular, the induction of a CTL response against cells harbouring the antigen is the only way to achieve its down-regulation by specific immunological means. However, in 5 the case of membrane-associated antigens, it is advantageous to induce a antibody response against the weak, cell-associated polypeptide antigen. However, when raising a humoral immune response against a weak cell-associated antigen it is preferred to substantially restrict the antibody response to 10 interaction with the parts of the antigen which are normally exposed to possible interaction with antibodies. Otherwise the result would most likely be the induction of an antibody response against parts of the antigen which is not normally engaging the humoral immune system, and this will in turn 15 increase the risk of inducing cross-reactivity with antigens not related to any pathology. One elegant way of obtaining this restriction is to perform nucleic acid vaccination with an analogue of the weak cell-associated antigen, where the extracellular part thereof is either unaltered or includes a T_H 20 epitope which does not substantially alter the 3D structure of the extracellular part of the antigen. As one possible alternative, immunization can be performed with both a CTL directed immunogen and a B-cell directed immunogen where the B-cell directed immunogen is substantially incapable of effecting 25 immunization against the intracellular part of the target antigen (the B-cell directed immunogen could e.g. lack any non-extracellular material from the antigen.

Induction of antibody responses can be achieved in a number of ways known to the person skilled in the art. For instance, the 30 at least one first analogue may comprise a part consisting of a modification of the structure of the cell-associated polypeptide antigen, said modification having as a result that immunization of the animal with the first analogue induces production of antibodies in the animal against the cell-asso-

25

ciated polypeptide antigen – this variant is as mentioned
above especially suited for nucleic acid vaccination. Alterna-
tively, the method of the invention can involve effecting
presentation to the animal's immune system of an immunogeni-
5 cally effective amount of at least one *second* analogue of the
cell-associated polypeptide antigen which contains such a
modification. A convenient way to achieve that the modifi-
cation has the desired antibody-inducing effect is to include
at least one second foreign T_H epitope in the second analogue,
10 *i.e.* a strategy like the one used for the first analogue.

In the cases where it is desired to also mount an effective
humoral immune response, it is advantageous that the first
and/or second analogue(s) comprise(s) a substantial fraction
of the cell-associated polypeptide antigen's B-cell epitopes,
15 especially a substantial fraction of such B-cell epitopes
which are extracellular in the naturally occurring form of the
antigen in the pertinent animal.

The above-discussed variations and modifications of the weak,
cell-associated polypeptide antigen can take different forms.
20 It is preferred that the variation and/or modification in-
volves amino acid substitution and/or deletion and/or inser-
tion and/or addition. These fundamental operations relating to
the manipulation of an amino acid sequence are intended to
cover both single-amino acid changes as well as operations
25 involving stretches of amino acids (*i.a.* shuffling of amino
acid stretches within the polypeptide antigen; this is espe-
cially interesting when the antigen is a true intracellular
antigen, since only considerations concerning preservation of
CTL epitopes are relevant). It will be understood, that the
30 introduction of e.g. one single amino acid insertion or dele-
tion may give rise to the emergence of a foreign T_H epitope in
the sequence of the analogue, *i.e.* the emergence of an MHC
Class II molecule binding sequence. However, in most situa-

tions it is preferable (and even necessary) to introduce a
known foreign T_H epitope, and such an operation will require
acid substitution and/or insertion (or sometimes addition in
the form of either conjugation to a carrier protein or provi-
5   sion of a fusion polypeptide by means of molecular biology
methods. It is preferred that the number of amino acid inser-
tions, deletions, substitutions or additions is at least 2,
such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17,
18, 19, 20, and 25 insertions, substitutions, additions or
10 deletions. It is furthermore preferred that the number of
amino acid substitutions is not in excess of 150, such as at
most 100, at most 90, at most 80, and at most 70. It is espe-
cially preferred that the number of substitutions, insertions,
deletions, or additions does not exceed 60, and in particular
15 the number should not exceed 50 or even 40. Most preferred is
a number of not more than 30.

Preferred embodiments of the invention includes modification
by introducing at least one foreign immunodominant T_H epitope.
It will be understood that the question of immune dominance of
20 a T-cell epitope depends on the animal species in question. As
used herein, the term "immunodominance" simply refers to
epitopes which in the vaccinated individual/population gives
rise to a significant immune response, but it is a well-known
fact that a T-cell epitope which is immunodominant in one
25 individual is not necessarily immunodominant in another indi-
vidual of the same species, even though it may be capable of
binding MHC-II molecules in the latter individual. True immune
dominant T_H epitopes are those which, independent of the
polypeptide wherein they form a subsequence, give rise to
30 activation of T_H cells - in other words, some T_H epitopes have,
as an intrinsic feature, the characteristic of substantially
never being cryptic since they are substantially always pro-
cessed by APCs and presented in the context of an MHC II
molecule on the surface of the APC.

27

Another important point is the issue of MHC restriction of T-cell epitopes. In general, naturally occurring T-cell epitopes are MHC restricted, *i.e.* a certain peptides constituting a T-cell epitope will only bind effectively to a subset of MHC

5 Class II molecules. This in turn has the effect that in most cases the use of one specific T-cell epitope will result in a vaccine component which is only effective in a fraction of the population, and depending on the size of that fraction, it can be necessary to include more T-cell epitopes in the same

10 molecule, or alternatively prepare a multi-component vaccine wherein the components are variants of the antigen which are distinguished from each other by the nature of the T-cell epitope introduced.

If the MHC restriction of the T-cells used is completely

15 unknown (for instance in a situation where the vaccinated animal has a poorly defined MHC composition), the fraction of the population covered by a specific vaccine composition can be determined by means of the following formula

20
$$f_{population} = 1 - \prod_{i=1}^{n} (1 - p_i) \tag{II}$$

-where p_i is the frequency in the population of responders to the ith foreign T-cell epitope present in the vaccine composition, and n is the total number of foreign T-cell epitopes in the vaccine composition. Thus, a vaccine composition contain-

25 ing 3 foreign T-cell epitopes having response frequencies in the population of 0.8, 0.7, and 0.6, respectively, would give

$$1 - 0.2 \times 0.3 \times 0.4 = 0.976$$

-*i.e.* 97.6 percent of the population will statistically mount an MHC-II mediated response to the vaccine.

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The above formula does not apply in situations where a more or less precise MHC restriction pattern of the peptides used is known. If, for instance a certain peptide only binds the human MHC-II molecules encoded by HLA-DR alleles DR1, DR3, DR5, and

5 DR7, then the use of this peptide together with another pep-tide which binds the remaining MHC-II molecules encoded by HLA-DR alleles will accomplish 100% coverage in the population in question. Likewise, if the second peptide only binds DR3 and DR5, the addition of this peptide will not increase the

10 coverage at all. If one bases the calculation of population response purely on MHC restriction of T-cell epitopes in the vaccine, the fraction of the population covered by a specific vaccine composition can be determined by means of the follow-ing formula:

15
$$f_{population} = 1 - \prod_{j=1}^{3} (1 - \varphi_j)^2 \qquad (III)$$

-wherein φ_j is the sum of frequencies in the population of allelic haplotypes encoding MHC molecules which bind any one of the T-cell epitopes in the vaccine and which belong to the jth of the 3 known HLA loci (DP, DR and DQ); in practice, it is

20 first determined which MHC molecules will recognize each T-cell epitope in the vaccine and thereafter these are listed by type (DP, DR and DQ) - then, the individual frequencies of the different listed allelic haplotypes are summed for each type, thereby yielding φ_1, φ_2, and φ_3.

25 It may occur that the value p_i in formula II exceeds the corre-sponding theoretical value π_i:

$$\pi_i = 1 - \prod_{j=1}^{3} (1 - v_j)^2 \qquad (IV)$$

-wherein v_j is the sum of frequencies in the population of allelic haplotype encoding MHC molecules which bind the ith T-cell epitope in the vaccine and which belong to the jth of the 3 known HLA loci (DP, DR and DQ). This means that in $1-\pi_i$ of the population is a frequency of responders of $f_{residual\_i} = (p_i - \pi_i)/(1-\pi_i)$. Therefore, formula III can be adjusted so as to yield formula V:

$$f_{population} = 1 - \prod_{j=1}^{3} (1 - \varphi_j)^2 + \left(1 - \prod_{i=1}^{n} (1 - f_{residual\_i})\right) \qquad (V)$$

-where the term $1-f_{residual-i}$ is set to zero if negative. It should be noted that formula V requires that all epitopes have been haplotype mapped against identical sets of haplotypes.

Therefore, when selecting T-cell epitopes to be introduced in the analogue, it is important to include all knowledge of the epitopes which is available: 1) The frequency of responders in the population to each epitope, 2) MHC restriction data, and 3) frequency in the population of the relevant haplotypes.

There exist a number of naturally occurring "promiscuous" T-cell epitopes which are active in a large proportion of individuals of an animal species or an animal population and these are preferably introduced in the vaccine thereby reducing the need for a very large number of different analogues in the same vaccine.

The promiscuous epitope can according to the invention be a naturally occurring human T-cell epitope such as epitopes from tetanus toxoid (e.g. the P2 and P30 epitopes), diphtheria toxoid, Influenza virus hemagluttinin (HA), and P. falciparum CS antigen.

Over the years a number of other promiscuous T-cell epitopes
have been identified. Especially peptides capable of binding a
large proportion of HLA-DR molecules encoded by the different
HLA-DR alleles have been identified and these are all possible

5   T-cell epitopes to be introduced in analogues used according
to the present invention. Cf. also the epitopes discussed in
the following references which are hereby all incorporated by
reference herein: WO 98/23635 (Frazer IH et al., assigned to
The University of Queensland); Southwood S et. al, 1998, J.

10 Immunol. **160**: 3363-3373; Sinigaglia F et al., 1988, Nature
336: 778-780; Rammensee HG et al., 1995, Immunogenetics **41**: 4
178-228; Chicz RM et al., 1993, J. Exp. Med **178**: 27-47; Hammer
J et al., 1993, Cell **74**: 197-203; and Falk K et al., 1994,
Immunogenetics **39**: 230-242. The latter reference also deals

15 with HLA-DQ and -DP ligands. All epitopes listed in these 5
references are relevant as candidate natural epitopes to be
used in the present invention, as are epitopes which share
common motifs with these.

Alternatively, the epitope can be any artificial T-cell epi-

20 tope which is capable of binding a large proportion of haplo-
types. In this context the pan DR epitope peptides ("PADRE")
described in WO 95/07707 and in the corresponding paper Alex-
ander J et al., 1994, Immunity 1: 751-761 (both disclosures
are incorporated by reference herein) are interesting candi-

25 dates for epitopes to be used according to the present inven-
tion. It should be noted that the most effective PADRE pep-
tides disclosed in these papers carry D-amino acids in the C-
and N-termini in order to improve stability when administered.
However, the present invention primarily aims at incorporating

30 the relevant epitopes as part of the modified antigen which
should then subsequently be broken down enzymatically inside
the lysosomal compartment of APCs to allow subsequent presen-
tation in the context of an MHC-II molecule and therefore it

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is not expedient to incorporate D-amino acids in the epitopes
used in the present invention.

One especially preferred PADRE peptide is the one having the
amino acid sequence AKFVAAWTLKAAA or an immunologically effec-
5 tive subsequence thereof. This, and other epitopes having the
same lack of MHC restriction are preferred T-cell epitopes
which should be present in the analogues used in the inventive
method. Such super-promiscuous epitopes will allow for the
most simple embodiments of the invention wherein only one
10 single analogue is presented to the vaccinated animal's immune
system.

The nature of the above-discussed variation/modification
preferably comprises that
    -    at least one first moiety is included in the first and/or
15   second analogue(s), said first moiety effecting targeting
         of the analogue to an antigen presenting cell (APC),
         and/or
    -    at least one second moiety is included in the first
         and/or second analogue(s), said second moiety stimulating
20   the immune system, and/or
    -    at least one third moiety is included in the first and/or
         second analogue(s), said third moiety optimizing presen-
         tation of the analogue to the immune system.

The functional and structural features relating these first,
25 second and third moieties will be discussed in the following:

They can be present in the form of side groups attached cova-
lently or non-covalently to suitable chemical groups in the
amino acid sequence of the cell-associated polypeptide antigen
or a subsequence thereof. This is to mean that stretches of
30 amino acid residues derived from the polypeptide antigen are
derivatized without altering the primary amino acid sequence,

or at least without introducing changes in the peptide bonds
between the individual amino acids in the chain.

The moieties can also be in the form of fusion partners to the
amino acid sequence derived from the cell-associated polypep-
5   tide antigen. In this connection it should be mentioned that
both possibilities include the option of conjugating the amino
acid sequence to a carrier, cf. the discussion of these below.
In other words, in the present context the term "fusion pro-
tein is not merely restricted to a fusion construct prepared
10 by means of expression of a DNA fragment encoding the con-
struct but also to a conjugate between two proteins which are
joined by means of a peptide bond in a subsequent chemical
reaction.

As mentioned above, the analogue can also include the intro-
15 duction of a first moiety which targets the analogue to an APC
or a B-lymphocyte. For instance, the first moiety can be a
specific binding partner for a B-lymphocyte specific surface
antigen or for an APC specific surface antigen. Many such
specific surface antigens are known in the art. For instance,
20 the moiety can be a carbohydrate for which there is a receptor
on the B-lymphocyte or the APC (e.g. mannan or mannose).
Alternatively, the second moiety can be a hapten. Also an
antibody fragment which specifically recognizes a surface
molecule on APCs or lymphocytes can be used as a first moiety
25 (the surface molecule can e.g. be an FCγ receptor of macro-
phages and monocytes, such as FCγRI or, alternatively any
other specific surface marker such as CD40 or CTLA-4). It
should be noted that all these exemplary targeting molecules
can be used as part of an adjuvant, cf. below. CD40 ligand,
30 antibodies against CD40, or variants thereof which bind CD40
will target the analogue to dendritic cells. At the same time,
recent results have shown that the interaction with the CD40
molecule renders the T_H cells unessential for obtaining a CTL

response. Hence, it is contemplated that the general use of
CD40 binding molecules as the first moiety (or as adjuvants,
cf. below) will enhance the CTL response considerably; in
fact, the use of such CD40 binding molecules as adjuvants and
5   "first moieties" in the meaning of the present invention is
believed to be inventive in its own right.

As an alternative or supplement to targeting the analogue to a
certain cell type in order to achieve an enhanced immune
response, it is possible to increase the level of responsive-
10 ness of the immune system by including the above-mentioned
second moiety which stimulates the immune system. Typical
examples of such second moieties are cytokines, heat-shock
proteins, and hormones, as well as effective parts thereof.

Suitable cytokines to be used according to the invention are
15 those which will normally also function as adjuvants in a
vaccine composition, e.g. interferon γ (IFN-γ), Flt3 ligand
(Flt3L), interleukin 1 (IL-1), interleukin 2 (IL-2), interleu-
kin 4 (IL-4), interleukin 6 (IL-6), interleukin 12 (IL-12),
interleukin 13 (IL-13), interleukin 15 (IL-15), and
20 granulocyte-macrophage colony stimulating factor (GM-CSF);
alternatively, the functional part of the cytokine molecule
may suffice as the second moiety. With respect to the use of
such cytokines as adjuvant substances, cf. the discussion
below.

25 Alternatively, the second moiety can be a toxin, such as
listeriolycin (LLO), lipid A and heat-labile enterotoxin.
Also, a number of mycobacterial derivatives such as MDP (mura-
myl dipeptide), CFA (complete Freund's adjuvant) and the
trehalose diesters TDM and TDE are interesting possibilities.

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According to the invention, suitable heat shock proteins used
as the second moiety can be HSP70, HSP90, HSC70, GRP94, and
calreticulin (CRT).

Also the possibility of introducing a third moiety which
5   enhances the presentation of the analogue to the immune system
is an important embodiment of the invention. The art has shown
several examples of this principle. For instance, it is known
that the palmitoyl lipidation anchor in the Borrelia burg-
dorferi protein OspA can be utilised so as to provide self-
10 adjuvating polypeptides (cf. e.g. WO 96/40718). It seems that
the lipidated proteins form up micelle-like structures with a
core consisting of the lipidation anchor parts of the polypep-
tides and the remaining parts of the molecule protruding
therefrom, resulting in multiple presentations of the anti-
15 genic determinants. Hence, the use of this and related ap-
proaches using different lipidation anchors (e.g. a myristyl
group, a farnesyl group, a geranyl-geranyl group, a GPI-an-
chor, and an N-acyl diglyceride group) are preferred embodi-
ments of the invention, especially since the provision of such
20 a lipidation anchor in a recombinantly produced protein is
fairly straightforward and merely requires use of e.g. a
naturally occurring signal sequence as a fusion partner for
the analogue. Another possibility is use of the C3d fragment
of complement factor C3 or C3 itself (cf. Dempsey et al.,
25 1996, Science 271, 348-350 and Lou & Kohler, 1998, Nature
Biotechnology 16, 458-462).

It is important to note that when attempting to use the method
of the invention against e.g. membrane bound polypeptide
antigens which are exposed to the extracellular compartment,
30 it is most preferred that the first and/or second analogue(s)
has/have substantially the overall tertiary structure of the
cell-associated polypeptide antigen. In the present specifica-
tion and claims this is intended to mean that the overall

tertiary structure of the *part* of the polypeptide antigen
which is extracellularly exposed is preserved, since, as
mentioned above, the tertiary structure of the obligate
intracellular polypeptides do not engage the humeral immune
5 system. In fact, as part of the vaccination strategy it is
often desired to avoid exposure to the extracellular compart-
ment of putative B-cell epitopes derived from intracellular
part of the polypeptide antigens; in this way, potentially
adverse effects caused by cross-reactivity with other antigens
10 can be minimized.

For the purposes of the present invention, it is however
sufficient if the variation/modification (be it an insertion,
addition, deletion or substitution) gives rise to a foreign T-
cell epitope and at the same time preserves a substantial
15 number of the CTL epitopes in the polypeptide antigen (and
sometimes also a substantial number of B-cell epitopes).

The following formula describes the constructs generally
covered by the invention:

$$(MOD_1)_{s1}(PAG_{e1})_{n1}(MOD_2)_{s2}(PAG_{e2})_{n2}\ldots(MOD_x)_{sx}(PAG_{ex})_{nx} \qquad (I)$$

20 -where $PAG_{e1}-PAG_{ex}$ are x CTL and/or B-Cell epitope containing
subsequences of the relevant polypeptide antigen which inde-
pendently are identical or non-identical and which may contain
or not contain foreign side groups, x is an integer ≥ 3, n1-nx
are x integers ≥ 0 (at least one is ≥ 1), MOD_1-MOD_x are x
25 modifications introduced between the preserved epitopes, and
s1-sx are x integers ≥ 0 (at least one is ≥ 1 if no side
groups are introduced in the sequences). Thus, given the
general functional restraints on the immunogenicity of the
constructs, the invention allows for all kinds of permutations
30 of the original antigen sequence, and all kinds of modifica-
tions therein. Thus, included in the invention are analogues

obtained by omission of parts of the polypeptide antigen sequence which e.g. exhibit adverse effects in vivo or omission of parts which are normally intracellular and thus could give rise to undesired immunological reactions, cf. the de-

5 tailed discussion below.

A further elaboration of the above principle include use of CTL and/or B-cell epitopes from more than one pathology-related antigen. For instance, there are several cancer related antigens that exert their oncogenic effects when they are in a

10 mutated form only – examples are mutated K-ras and P53 which both are crucial proteins in normal cell cycle regulation and which both are expression products in most normal cells. In some cases, CTLs have been shown to recognise mutated peptides from these antigens. It is therefore important that the immune

15 system responds to te mutated peptide only, and not to the unmutated parts, if antigen specific immunotherapy is instigated.

We have devised a strategy whereby sequences of 8-25 amino acids of such disease-related proteins could be used as fur-

20 ther epitopes in an AutoVac construct – in preferred embodiments, the introduced epitopes would at the same time provide for the emergence of T_H epitopes in the final construct, cf. the discussion above. The epitopes used for this purpose would be those which comprise the mutated region of the disease-

25 related protein. By using such an approach, it would be possible to generate CTLs (and possibly antibodies, where applicable) against only the mutated form of the disease-related antigen. In the cases where the disease-related antigen provides for the emergence of a T_H epitope, the use of a truly

30 foreign T_H epitope could be completely omitted. An embodiment of this principle could e.g. be vaccination with a nucleic acid vaccine which encode an analogue of a polypeptide antigen (e.g. Her2 or PSM) wherein has been introduced at least one T_H

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epitope and at least one peptide derived from another disease-
related antigen (e.g. a peptide from the mutated part of an
oncogenic protein). In a preferred embodiment, the at least
one T_H epitope is introduced as a consequence of the introduc-
5 tion of the peptide.

It is furthermore preferred that the variation and/or modifi-
cation includes duplication, when applicable, of the at least
one B-cell epitope, or of at least one CTL epitope of the
cell-associated polypeptide antigen. This strategy will give
10 the result that multiple copies of preferred epitopic regions
are presented to the immune system and thus maximizing the
probability of an effective immune response. Hence, this
embodiment of the invention utilises multiple presentations of
epitopes derived from the polypeptide antigen (i.e. formula I
15 wherein at least one B-cell epitope is present in two posi-
tions).

This effect can be achieved in various ways, e.g. by simply
preparing fusion polypeptides comprising the structure $(PAG)_m$,
where m is an integer $\geq$ 2 and then introduce the modifications
20 discussed herein in at least one of the polypeptide antigen
sequences.

An alternative embodiment of the invention which also results
in the preferred presentation of multiple (e.g. at least 2)
copies of the important epitopic regions of the antigen to the
25 immune system is the covalent coupling of the antigen, subse-
quence or variants thereof to certain molecules. For instance,
polymers can be used, e.g. carbohydrates such as dextran, cf.
e.g. Lees A et al., 1994, Vaccine 12: 1160-1166; Lees A et
al., 1990, J Immunol. 145: 3594-3600, but also mannose and
30 mannan are useful alternatives. Integral membrane proteins
from e.g. E. coli and other bacteria are also useful conjuga-
tion partners. The traditional carrier molecules such as

keyhole limpet haemocyanin (KLH), tetanus toxoid, diphtheria
toxoid, and bovine serum albumin (BSA) are also preferred and
useful conjugation partners.

Maintenance of the sometimes advantageous substantial fraction
5   of B-cell epitopes or even the overall tertiary structure of a
protein which is subjected to modification as described herein
can be achieved in several ways. One is simply to prepare a
polyclonal antiserum directed against the polypeptide antigen
(e.g. an antiserum prepared in a rabbit) and thereafter use
10 this antiserum as a test reagent (e.g. in a competitive ELISA)
against the modified proteins which are produced. Modified
versions (analogues) which react to the same extent with the
antiserum as does the polypeptide antigen must be regarded as
having the same overall tertiary structure as the polypeptide
15 antigen whereas analogues exhibiting a limited (but still
significant and specific) reactivity with such an antiserum
are regarded as having maintained a substantial fraction of
the original B-cell epitopes.

Alternatively, a selection of monoclonal antibodies reactive
20 with distinct epitopes on the polypeptide antigen can be
prepared and used as a test panel. This approach has the
advantage of allowing 1) an epitope mapping of the polypeptide
antigen in question and 2) a mapping of the epitopes which are
maintained in the analogues prepared.

25 Of course, a third approach would be to resolve the 3-dimen-
sional structure of the polypeptide antigen or of a biologi-
cally active truncate thereof (cf. above) and compare this to
the resolved three-dimensional structure of the analogues
prepared. Three-dimensional structure can be resolved by the
30 aid of X-ray diffraction studies and NMR-spectroscopy. Further
information relating to the tertiary structure can to some
extent be obtained from circular dichroism studies which have

the advantage of merely requiring the polypeptide in pure form
(whereas X-ray diffraction requires the provision of crystal-
lized polypeptide and NMR requires the provision of isotopic
variants of the polypeptide) in order to provide useful infor-
5   mation about the tertiary structure of a given molecule.
However, ultimately X-ray diffraction and/or NMR are necessary
to obtain conclusive data since circular dichroism can only
provide indirect evidence of correct 3-dimensional structure
via information of secondary structure elements.

10 In essence there are at present three feasible ways of obtain-
ing the presentation of the relevant epitopes to the immune
system: Traditional sub-unit vaccination with polypeptide
antigens, administration of a genetically modified live vac-
cine, and nucleic acid vaccination. These three possibilities
15 will be discussed separately in the following:

Polypeptide vaccination

This entails administration to the animal in question of an
immunogenically effective amount of the at least one first
analogue, and, when relevant, administration of an immunologi-
20 cally effective amount of the at least one second analogue.
Preferably, the at least one first and/or second analogue(s)
is/are formulated together with a pharmaceutically and immuno-
logically acceptable carrier and/or vehicle and, optionally an
adjuvant.

25 When effecting presentation of the analogue to an animal's
immune system by means of administration thereof to the ani-
mal, the formulation of the polypeptide follows the principles
generally acknowledged in the art.

Preparation of vaccines which contain peptide sequences as
30 active ingredients is generally well understood in the art, as

exemplified by US Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as inject-
5 ables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients
10 are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if de-sired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the
15 vaccines; cf. the detailed discussion of adjuvants below.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously, intradermally, subdermally or intramuscularly. Additional formulations which are suitable for other modes of administration include suppos-
20 itories and, in some cases, oral, buccal, sublinqual, intraperitoneal, intravaginal, anal and intracranial formula-tions. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides; such suppositories may be formed from mixtures containing the
25 active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipi-ents as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellu-lose, magnesium carbonate, and the like. These compositions
30 take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and con-tain 10-95% of active ingredient, preferably 25-70%. For oral formulations, cholera toxin is an interesting formulation partner (and also a possible conjugation partner).

The polypeptides may be formulated into the vaccine as neutral
or salt forms. Pharmaceutically acceptable salts include acid
addition salts (formed with the free amino groups of the
peptide) and which are formed with inorganic acids such as,
5 for example, hydrochloric or phosphoric acids, or such organic
acids as acetic, oxalic, tartaric, mandelic, and the like.
Salts formed with the free carboxyl groups may also be derived
from inorganic bases such as, for example, sodium, potassium,
ammonium, calcium, or ferric hydroxides, and such organic
10 bases as isopropylamine, trimethylamine, 2-ethylamino ethanol,
histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the
dosage formulation, and in such amount as will be therapeuti-
cally effective and immunogenic. The quantity to be adminis-
15 tered depends on the subject to be treated, including, e.g.,
the capacity of the individual's immune system to mount an
immune response, and the degree of protection desired. Suit-
able dosage ranges are of the order of several hundred micro-
grams active ingredient per vaccination with a preferred range
20 from about 0.1 µg to 2000 µg (even though higher amounts in
the 1-10 mg range are contemplated), such as in the range from
about 0.5 µg to 1000 µg, preferably in the range from 1 µg to
500 µg and especially in the range from about 10 µg to 100 µg.
Suitable regimens for initial administration and booster shots
25 are also variable but are typified by an initial administra-
tion followed by subsequent inoculations or other administra-
tions.

The manner of application may be varied widely. Any of the
conventional methods for administration of a vaccine are
30 applicable. These include oral application on a solid physio-
logically acceptable base or in a physiologically acceptable
dispersion, parenterally, by injection or the like. The dosage
of the vaccine will depend on the route of administration and

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will vary according to the age of the person to be vaccinated
and the formulation of the antigen.

Some of the polypeptides of the vaccine are sufficiently
immunogenic in a vaccine, but for some of the others the
5 immune response will be enhanced if the vaccine further com-
prises an adjuvant substance. It is especially preferred to
use an adjuvant which can be demonstrated to facilitate break-
ing of the autotolerance to autoantigens.

Various methods of achieving adjuvant effect for the vaccine
10 are known. General principles and methods are detailed in "The
Theory and Practical Application of Adjuvants", 1995, Duncan
E.S. Stewart-Tull (ed.), John Wiley & Sons Ltd, ISBN 0-471-
95170-6, and also in "Vaccines: New Generationn Immunological
Adjuvants", 1995, Gregoriadis G et al. (eds.), Plenum Press,
15 New York, ISBN 0-306-45283-9, both of which are hereby incor-
porated by reference herein.

Preferred adjuvants facilitate uptake of the vaccine molecules
by APCs, such as dendritic cells, and activate these. Non-
limiting examples are selected from the group consisting of an
20 immune targeting adjuvant; an immune modulating adjuvant such
as a toxin, a cytokine, and a mycobacterial derivative; an oil
formulation; a polymer; a micelle forming adjuvant; a saponin;
an immunostimulating complex matrix (ISCOM matrix); a par-
ticle; DDA; aluminium adjuvants; DNA adjuvants; γ-inulin; and
25 an encapsulating adjuvant. In general it should be noted that
the disclosures above which relate to compounds and agents
useful as first, second and third moieties in the analogues
also refer *mutatis mutandis* to their use in the adjuvant of a
vaccine of the invention.

30 The application of adjuvants include use of agents such as
aluminum hydroxide or phosphate (alum), commonly used as 0.05

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to 0.1 percent solution in buffered saline, admixture with
synthetic polymers of sugars (e.g. Carbopol®) used as 0.25
percent solution, aggregation of the protein in the vaccine by
heat treatment with temperatures ranging between 70° to 101°C

5 for 30 second to 2 minute periods respectively and also aggre-
gation by means of cross-linking agents are possible. Aggrega-
tion by reactivation with pepsin treated antibodies (Fab
fragments) to albumin, mixture with bacterial cells such as C.
parvum or endotoxins or lipopolysaccharide components of gram-

10 negative bacteria, emulsion in physiologically acceptable oil
vehicles such as mannide mono-oleate (Aracel A) or emulsion
with 20 percent solution of a perfluorocarbon (Fluosol-DA)
used as a block substitute may also be employed. Admixture
with oils such as squalene and IFA is also preferred.

15 According to the invention DDA (dimethyldioctadecylammonium
bromide) is an interesting candidate for an adjuvant as is DNA
and γ-inulin, but also Freund's complete and incomplete adju-
vants as well as quillaja saponins such as QuilA and QS21 are
interesting. Further possibilities are monophosphoryl lipid A

20 (MPL), and the above mentioned C3 and C3d.

Liposome formulations are also known to confer adjuvant ef-
fects, and therefore liposome adjuvants are preferred accor-
ding to the invention.

Also immunostimulating complex matrix type (ISCOM® matrix)
25 adjuvants are preferred choices according to the invention,
especially since it has been shown that this type of adjuvants
are capable of up-regulating MHC Class II expression by APCs.
An ISCOM® matrix consists of (optionally fractionated) sapo-
nins (triterpenoids) from Quillaja saponaria, cholesterol, and

30 phospholipid. When admixed with the immunogenic protein, the
resulting particulate formulation is what is known as an ISCOM
particle where the saponin constitutes 60-70% w/w, the choles-

terol and phospholipid 10-15% w/w, and the protein 10-15% w/w.
Details relating to composition and use of immunostimulating
complexes can e.g. be found in the above-mentioned text-books
dealing with adjuvants, but also Morein B et al., 1995, Clin.
5 Immunother. 3: 461-475 as well as Barr IG and Mitchell GF,
1996, Immunol. and Cell Biol. 74: 8-25 (both incorporated by
reference herein) provide useful instructions for the prepara-
tion of complete immunostimulating complexes.

Another highly interesting (and thus, preferred) possibility
10 of achieving adjuvant effect is to employ the technique de-
scribed in Gosselin et al., 1992 (which is hereby incorporated
by reference herein). In brief, the presentation of a relevant
antigen such as an antigen of the present invention can be
enhanced by conjugating the antigen to antibodies (or antigen
15 binding antibody fragments) against the Fcγ receptors on
monocytes/macrophages. Especially conjugates between antigen
and anti-FcγRI have been demonstrated to enhance immunoge-
nicity for the purposes of vaccination.

Other possibilities involve the use of the targeting and
20 immune modulating substances (i.a. cytokines) mentioned above
as candidates for the first and second moieties in the modi-
fied analogues. In this connection, also synthetic inducers of
cytokines like poly I:C are possibilities.

Suitable mycobacterial derivatives are selected from the group
25 consisting of muramyl dipeptide, complete Freund's adjuvant,
RIBI, and a diester of trehalose such as TDM and TDE.

Suitable immune targeting adjuvants are selected from the
group consisting of CD40 ligand and CD40 antibodies or speci-
fically binding fragments thereof (cf. the discussion above),
30 mannose, a Fab fragment, and CTLA-4.

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Suitable polymer adjuvants are selected from the group con-
sisting of a carbohydrate such as dextran, PEG, starch, man-
nan, and mannose; a plastic polymer; and latex such as latex
beads.

5 Yet another interesting way of modulating an immune response
   is to include the immunogen (optionally together with adju-
   vants and pharmaceutically acceptable carriers and vehicles)
   in a "virtual lymph node" (VLN) (a proprietary medical device
   developed by ImmunoTherapy, Inc., 360 Lexington Avenue, New
10 York, NY 10017-6501). The VLN (a thin tubular device) mimics
   the structrue and function of a lymph node. Insertion of a VLN
   under the skin creates a site of sterile inflammation with an
   upsurge of cytokines and chemokines. T- and B-cells as well as
   APCs rapidly respond to the danger signals, home to the in-
15 flamed site and accumulate inside the porous matrix of the
   VLN. It has been shown that the necessary antigen dose re-
   quired to mount an immune response to an antigen is reduced
   when using the VLN and that immune protection conferred by
   vaccination using a VLN surpassed conventional immunization
20 using Ribi as an adjuvant. The technology is *i.a.* described
   briefly in Gelber C *et al.*, 1998, "Elicitation of Robust
   Cellular and Humoral Immune Responses to Small Amounts of
   Immunogens Using a Novel Medical Device Designated the Virtual
   Lymph Node", in: "From the Laboratory to the Clinic, Book of
25 Abstracts, October 12th – 15th 1998, Seascape Resort, Aptos,
   California".

Recent findings have demonstrated that the co-administration
of H2 agonists enhances the in-tumour survival of Natural
Killer Cells and CTLs. Hence, it is also contemplated to
30 include H2 agonists as adjuvants in the methods of the inven-
   tion.

It is expected that the vaccine should be administered at
least once a year, such as at least 1, 2, 3, 4, 5, 6, and 12
times a year. More specifically, 1-12 times per year is ex-
pected, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 times
5 a year to an individual in need thereof. It has previously
been shown that the memory immunity induced by the use of the
preferred autovaccines according to the invention is not
permanent, and therefor the immune system needs to be periodi-
cally challenged with the analogues.

10 Due to genetic variation, different individuals may react with
immune responses of varying strength to the same polypeptide.
Therefore, the vaccine according to the invention may comprise
several different polypeptides in order to increase the immune
response, cf. also the discussion above concerning the choice
15 of foreign T-cell epitope introductions. The vaccine may
comprise two or more polypeptides, where all of the polypep-
tides are as defined above.

The vaccine may consequently comprise 3-20 different modified
or unmodified polypeptides, such as 3-10 different polypep-
20 tides. However, normally the number of peptides will be sought
kept to a minimum such as 1 or 2 peptides.

Live vaccines

The second alternative for effecting presentation to the
immune system is the use of live vaccine technology. In live
25 vaccination, presentation to the immune system is effected by
administering, to the animal, a non-pathogenic microorganism
which has been transformed with a nucleic acid fragment enco-
ding the necessary epitopic regions or a complete 1st and/or 2nd
analogue. Alternatively, the microorganism is transformed with
30 a vector incorporating such a nucleic acid fragment. The non-
pathogenic microorganism can be any suitable attenuated bacte-

rial strain (attenuated by means of passaging or by means of
removal of pathogenic expression products by recombinant DNA
technology), e.g. *Mycobacterium bovis* BCG., non-pathogenic
Streptococcus spp., *E. coli*, *Salmonella* spp., *Vibrio cholerae*,
5 *Shigella*, etc. Reviews dealing with preparation of state-of-
the-art live vaccines can e.g. be found in Saliou P, 1995,
Rev. Prat. 45: 1492-1496 and Walker PD, 1992, Vaccine 10:
977-990, both incorporated by reference herein. For details
about the nucleic acid fragments and vectors used in such live
10 vaccines, cf. the discussion below.

As for the polypeptide vaccine, the T_H epitope and/or the first
and/or second and/or third moieties can, if present, be in the
form of fusion partners to the amino acid sequence derived
from the cell-associated polypeptide antigen.

15 As an alternative to bacterial live vaccines, the nucleic acid
fragment of the invention discussed below can be incorporated
in a non-virulent viral vaccine vector. One possibility is a
pox virus such as vaccinia, MVA (modified Vaccinia virus),
canary pox, avi-pox, and chicken pox etc. Alternatively, a
20 herpes simplex virus variant can be used.

Normally, the non-pathogenic microorganism or virus is admin-
istered only once to the animal, but in certain cases it may
be necessary to administer the microorganism more than once in
a lifetime.

25 Also, the microorganism can be transformed with nucleic
acid(s) containing regions encoding the 1st, 2nd and/or 3rd
moieties, e.g. in the form of the immunomodulating substances
described above such as the cytokines discussed as useful
adjuvants. A preferred version of this embodiment encompasses
30 having the coding region for the analogue and the coding
region for the immunomodulator in different open reading

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frames or at least under the control of different promoters. Thereby it is avoided that the analogue or epitopes are produced as fusion partners to the immunomodulator. Alternatively, two distinct nucleotide fragments can be used as
5 transforming agents.

Nucleic acid vaccination

As an alternative to classic administration of a peptide-based vaccine, the technology of nucleic acid vaccination (also known as "nucleic acid immunisation", "genetic immunisation",
10 "gene immunisation" and "DNA vaccination) offers a number of attractive features.

First, in contrast to the traditional vaccine approach, nucleic acid vaccination does not require resource consuming large-scale production of the immunogenic agent (e.g. in the
15 form of industrial scale fermentation of microorganisms producing the analogues necessary in polypeptide vaccination). Furthermore, there is no need to device purification and refolding schemes for the immunogen. And finally, since nucleic acid vaccination relies on the biochemical apparatus of
20 the vaccinated individual in order to produce the expression product of the nucleic acid introduced, the optimum posttranslational processing of the expression product is expected to occur; this is especially important in the case of autovaccination, since, as mentioned above, a significant
25 fraction of the original B-cell epitopes should be preserved in the analogues derived from extracellularly exposed polypeptide sequences, and since B-cell epitopes in principle can be constituted by parts of any (bio)molecule (e.g. carbohydrate, lipid, protein etc.). Therefore, native glycosylation and
30 lipidation patterns of the immunogen may very well be of importance for the overall immunogenicity and this is best ensured by having the host producing the immunogen.

Hence, an important embodiment of the method of the invention
involves that presentation is effected by *in vivo* introducing,
into the APC, at least one nucleic acid fragment which encodes
and expresses the at least one CTL epitope and/or the at least

5　one B-cell epitope, and the at least one first foreign T_H
epitope (an alternative encompasses administration of at least
2 distinct nucleic acid fragments, where one encodes the at
least one CTL epitope and the other encodes the at least one
foreign T_H epitope). Preferably, this is done by using a nu-

10　cleic acid fragment which encodes and expresses the above-
discussed first analogue. If the first analogue is equipped
with the above-detailed T_H epitopes and/or first and/or second
and/or third moieties, these are then present in the form of
fusion partners to the amino acid sequence derived from the

15　cell-associated polypeptide antigen, the fusion construct
being encoded by the nucleic acid fragment.

As for the traditional vaccination approach, the nucleic acid
vaccination can be combined with *in vivo* introduction, into
the APC, of at least one nucleic acid fragment encoding and

20　expressing the second analogue. The considerations pertaining
to 1st, 2nd and 3rd moieties and T_H epitopes apply also here.

In this embodiment, the introduced nucleic acid is preferably
DNA which can be in the form of naked DNA, DNA formulated with
charged or uncharged lipids, DNA formulated in liposomes, DNA

25　included in a viral vector, DNA formulated with a
transfection-facilitating protein or polypeptide, DNA formu-
lated with a targeting protein or polypeptide, DNA formulated
with Calcium precipitating agents, DNA coupled to an inert
carrier molecule, and DNA formulated with an adjuvant. In this

30　context it is noted that practically all considerations per-
taining to the use of adjuvants in traditional vaccine formu-
lation apply for the formulation of DNA vaccines. Hence, all
disclosures herein which relate to use of adjuvants in the

context of polypeptide based vaccines apply *mutatis mutandis*
to their use in nucleic acid vaccination technology. The same
holds true for other considerations relating to formulation
and mode and route of administration and, hence, also these
5 considerations discussed above in connection with a tradi-
tional vaccine apply *mutatis mutandis* to their use in nucleic
acid vaccination technology.

One especially preferred type of formulation of nucleic acid
vaccines are microparticles containing the DNA. Suitable
10 microparticles are e.g. described in WO 98/31398.

Furthermore, the nucleic acid(s) used as an immunization agent
can contain regions encoding the 1st, 2nd and/or 3rd moieties,
e.g. in the form of the immunomodulating substances described
above such as the cytokines discussed as useful adjuvants. A
15 preferred version of this embodiment encompasses having the
coding region for the analogue and the coding region for the
immunomodulator in different open reading frames or at least
under the control of different promoters. Thereby it is avoid-
ed that the analogue or epitope is produced as a fusion part-
20 ner to the immunomodulator. Alternatively, two distinct
nucleotide fragments can be used, but this is less preferred
because of the advantage of ensured co-expression when having
both coding regions included in the same molecule.

Under normal circumstances, the nucleic acid of the vaccine is
25 introduced in the form of a vector wherein expression is under
control of a viral promoter. For more detailed discussions of
vectors according to the invention, cf. the discussion below.
Also, detailed disclosures relating to the formulation and use
of nucleic acid vaccines are available, cf. Donnelly JJ et al,
30 1997, Annu. Rev. Immunol. 15: 617-648 and Donnelly JJ et al.,
1997, Life Sciences 60: 163-172. Both of these references are
incorporated by reference herein.

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An important part of the invention pertains to a novel method
for selecting an appropriate immunogenic analogue of a cell-
associated polypeptide antigen which is weakly immunogenic or
non-immunogenic in an animal, said immunogenic analogue being
5 capable of inducing a CTL response in the animal against cells
displaying an MHC Class I molecule bound to an epitope derived
from the cell-associated polypeptide antigen. This method
comprises the steps of

a)   identifying at least one subsequence of the amino acid
10   sequence of the cell-associated polypeptide antigen,
          where said subsequence does not contain known or pre-
          dicted CTL epitopes,

b)   preparing at least one putatively immunogenic analogue of
          the cell-associated polypeptide antigen by introducing,
15   in the amino acid sequence of the cell-associated
          polypeptide antigen, at least one T_H epitope foreign to
          the animal in a position within the at least one subse-
          quence identified in step a), and

c)   selecting the/those analogues prepared in step b) which
20   are verifiably capable of inducing a CTL response in the
          animal.

Alternatively, the above selection method involves the prepa-
ration of a nucleic acid fragment for nucleic acid vaccination
purposes. In that situation, it is required that the encoded
25 peptide includes at least one T_H epitope.

When the analogue is derived from an antigen which is exposed
to the extracellular phase, it is preferred that the subse-
quence identified in step a) further does not contain cysteine
residues, or, alternatively, that the T_H epitope introduced in
30 step b) does not substantially alter the pattern of cysteine
residues. This approach facilitates the preservation of spa-
tial B-cell epitopes in the resulting construct which are

similar to the B-cell epitopes in the weak, cell-associated
polypeptide antigen.

For the same reasons it is preferred that the subsequence
identified in step a) further does not contain known or pre-
5 dicted glycosylation sites, or, alternatively, wherein the T_H
epitope introduced in step b) does not substantially alter the
glycosylation pattern.

Certain of the weak, cell-associated polypeptide antigens
exert undesired effects by having a pathophysiological role.
10 It is desired that these effects are not exerted by the vacci-
nation constructs, and therefore it is preferred that the
subsequence identified in step a) contributes significantly to
a pathophysiological effect exerted by the cell-associated
polypeptide antigen, and that the introduction in step b) of
15 the foreign T_H epitope reduces or abolishes said pathophysiolo-
gical effect. An example of this approach is to remove the
active site in an enzyme, hormone or cytokine and exchange
this with the foreign T_H epitope.

Another important consideration pertains to the question of
20 immunological cross-reactivity of the vaccine's polypeptide
product with other self-proteins which are not related to a
pathology. Such cross-reactivity should preferably be avoided
and hence an important embodiment of this method of the inven-
tion is one where the subsequence identified in step a) is
25 homologous to an amino acid sequence of a different protein
antigen of the animal, and where the introduction of the T_H
epitope in step b) substantially removes the homology.

Related to this embodiment is an embodiment where any amino
acid sequences which 1) are not normally exposed to the
30 extracellular phase and 2) which may constitute B-cell epi-
topes of the weak, cell-associated polypeptide antigen, are

not preserved in the analogue. This can be achieved by ex-
changing such amino acid sequences with T_H epitopes which do
not constitute B-cell epitopes, by completely removing them,
or by partly removing them.

5   On the other hand, it is preferred that any "true" B-cell
epitopes of the weak cell-associated polypeptide antigen are
preserved to a high degree, and therefore an important embodi-
ment of the selection method of the invention involves that
the introduction in step b) of the foreign T_H epitope results
10 in preservation of a substantial fraction of B-cell epitopes
of the cell-associated polypeptide antigen. It is especially
preferred that the analogue preserves the overall tertiary
structure of the cell-associated polypeptide antigen.

The preparation in step b) is preferably accomplished by
15 molecular biological means or by means of solid or liquid
phase peptide synthesis. Shorter peptides are preferably
prepared by means of the well-known techniques of solid- or
liquid-phase peptide synthesis. However, recent advances in
this technology has rendered possible the production of full-
20 length polypeptides and proteins by these means, and therefore
it is also within the scope of the present invention to pre-
pare the long constructs by synthetic means.

After having identified the useful analogues according to the
above-discussed method, it is necessary to produce the ana-
25 logue in larger scale. The polypeptides are prepared according
to methods well-known in the art.

This can be done by molecular biological means comprising a
first step of preparing a transformed cell by introducing,
into a vector, a nucleic acid sequence encoding an analogue
30 which has been selected according to the method and transform-
ing a suitable host cell with the vector. The next step is to

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culture the transformed cell under conditions facilitating the
expression of the nucleic acid fragment encoding the analogue
of the cell-associated antigen, and subsequently recovering
the analogue from the culture supernatant or directly from the
5 cells, e.g. in the form of a lysate). Alternatively, the
analogue can be prepared by large-scale solid or liquid phase
peptide synthesis, cf. above.

Finally, the product can, depending on the cell chosen as a
host cell or the synthesis method used, be subjected to arti-
10 ficial post-translational modifications. These can be refol-
ding schemes known in the art, treatment with enzymes (in
order to obtain glycosylation or removal of undesired fusion
partners, chemical modifications (again glycosylation is a
possibility), and conjugation, e.g. to traditionally carrier
15 molecules.

It should be noted that preferred analogues of the invention
(and also the relevant analogues used in the methods of the
invention) comprise modifications which results in a polypep-
tide having a sequence identity of at least 70% with the
20 polypeptide antigen or with a subsequence thereof of at least
10 amino acids in length. Higher sequence identities are
preferred, e.g. at least 75% or even at least 80% or 85%. The
sequence identity for proteins and nucleic acids can be calcu-
lated as $(N_{ref}-N_{dif}) \cdot 100/N_{ref}$, wherein N_{dif} is the total number of
25 non-identical residues in the two sequences when aligned and
wherein N_{ref} is the number of residues in one of the sequences.
Hence, the DNA sequence AGTCAGTC will have a sequence identity
of 75% with the sequence AATCAATC ($N_{dif}=2$ and $N_{ref}=8$).

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Specific exemplary targets for the method of the invention

As discussed above, preferred weak, cell-associated polypep-
tide antigens are tumour-associated antigens. A non-limiting
list of these are given in the following table.

Antigen	Reference
5 alpha reductase	Délos S, Carsol JL, Fina F, Raynaud JP, Martin PM. 5alpha-reductase and 17beta-hydroxysteroid dehydrogenase expression in epithelial cells from hyperplastic and malignant human prostate. Int J Cancer 1998 Mar 16 75:6 840-6
α-fetoprotein	Esteban C, Terrier P, Frayssinet C, Uriel J. Expression of the alpha-fetoprotein gene in human breast cancer. Tumour Biol 1996 17:5 299-305
AM-1	Harada Y, Ohuchi N, Masuko T, Funaki Y, Mori S, Satomi S, Hashimoto Y. Characterization of a new breast cancer-associated antigen and its relationship to MUC1 and TAG-72 antigens. Tohoku J Exp Med 1996 Nov 180:3 273-88
APC	Dihlmann S, Amler LC, Schwab M, Wenzel A. Variations in the expression of the adenomatous polyposis coli (APC) tumor suppressor gene in human cancer cell lines of different tissue origin. Oncol Res 1997 9:3 119-27
APRIL	LE, Sordat B, Rimoldi D, Tschopp J. APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. J Exp Med 1998 Sep 21 188:6 1185-90
BAGE	Böel P, Wildmann C, Sensi ML, Brasseur R, Renauld J-C, Coulie P, Boon T, and Van der Bruggen P. BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic lymphocytes. Immunity 1995, 2: 167-175.
β-catenin	Hugh TJ, Dillon SA, O'Dowd G, Getty B, Pignatelli M, Poston GJ, Kinsella AR. beta-catenin expression in primary and metastatic colorectal carcinoma. Int J Cancer 1999 Aug 12 82:4 504-11
Bcl2	Koty PP, Zhang H, Levitt ML. Antisense bcl-2 treatment increases programmed cell death in non-small cell lung cancer cell lines. Lung Cancer 1999 Feb 23:2 115-27
bcr-abl (b3a2)	Verfaillie CM, Bhatia R, Miller W, Mortari F, Roy V, Burger S, McCullough J, Stieglbauer K, Dewald G, Heimfeld S, Miller JS, McGlave PB. BCR/ABL-negative primitive progenitors suitable for transplantation can be selected from the marrow of most early-chronic phase

Antigen	Reference
	but not accelerated-phase chronic myelogenous leukemia patients. Blood 1996 Jun 1 87:11 4770-9
CA-125	Bast RC Jr, Xu FJ, Yu YH, Barnhill S, Zhang Z, Mills GB. CA 125: the past and the future. Int J Biol Markers 1998 Oct-Dec 13:4 179-87
CASP-8 / FLICE	Mandruzzato S, Brasseur F, Andry G, Boon T, van der Bruggen P., A CASP-8 mutation recognized by cytolytic T lymphocytes on a human head and neck carcinoma. J Exp Med 1997 Aug 29 186:5 785-93.
Cathepsins	Thomssen C, Schmitt M, Goretzki L, Oppelt P, Pache L, Dettmar P, Jänicke F, Graeff H. Prognostic value of the cysteine proteases cathepsins B and cathepsin L in human breast cancer. Clin Cancer Res 1995 Jul 1:7 741-6
CD19	Scheuermann RH, Racila E. CD19 antigen in leukemia and lymphoma diagnosis and immunotherapy. Leuk Lymphoma 1995 Aug 18:5-6 385-97
CD20	Knox SJ, Goris ML, Trisler K, Negrin R, Davis T, Liles TM, Grillo-López A, Chinn P, Varns C, Ning SC, Fowler S, Deb N, Becker M, Marquez C, Levy R. Yttrium-90-labeled anti-CD20 monoclonal antibody therapy of recurrent B-cell lymphoma. Clin Cancer Res 1996 Mar 2:3 457-70
CD21	Shubinsky G, Schlesinger M, Polliack A, Rabinowitz R. Pathways controlling the expression of surface CD21 (CR2) and CD23 (Fc(epsilon)IIR) proteins in human malignant B cells. Leuk Lymphoma 1997 May 25:5-6 521-30
CD23	Shubinsky G, Schlesinger M, Polliack A, Rabinowitz R. Pathways controlling the expression of surface CD21 (CR2) and CD23 (Fc(epsilon)IIR) proteins in human malignant B cells. Leuk Lymphoma 1997 May 25:5-6 521-30
CD22	French RR, Penney CA, Browning AC, Stirpe F, George AJ, Glennie MJ. Delivery of the ribosome-inactivating protein, gelonin, to lymphoma cells via CD22 and CD38 using bispecific antibodies. Br J Cancer 1995 May 71:5 986-94
CD33	Nakase K, Kita K, Shiku H, Tanaka I, Nasu K, Dohy H, Kyo T, Tsutani H, Kamada N. Myeloid antigen, CD13, CD14, and/or CD33 expression is restricted to certain lymphoid neoplasms. Am J Clin Pathol 1996 Jun 105:6 761-8
CD35	Yamakawa M, Yamada K, Tsuge T, Ohrui H, Ogata T, Dobashi M, Imai Y. Protection of thyroid cancer cells by complement-regulatory factors. Cancer 1994 Jun 1 73:11 2808-17

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Antigen	Reference
CD44	Naot D, Sionov RV, Ish-Shalom D. CD44: structure, function, and association with the malignant process. Adv Cancer Res 1997 71: 241-319
CD45	Buzzi M, Lu L, Lombardi AJ Jr, Posner MR, Brautigan DL, Fast LD, Frackelton AR Jr. Differentiation-induced changes in protein-tyrosine phosphatase activity and commensurate expression of CD45 in human leukemia cell lines. Cancer Res 1992 Jul 15 52:14 4027-35
CD46	Yamakawa M, Yamada K, Tsuge T, Ohrui H, Ogata T, Dobashi M, Imai Y. Protection of thyroid cancer cells by complement-regulatory factors. Cancer 1994 Jun 1 73:11 2808-17
CD5	Stein R, Witz IP, Ovadia J, Goldenberg DM, Yron I. CD5+ B cells and naturally occurring autoantibodies in cancer patients. Clin Exp Immunol 1991 Sep 85:3 418-23
CD52	Ginaldi L, De Martinis M, Matutes E, Farahat N, Morilla R, Dyer MJ, Catovsky D. Levels of expression of CD52 in normal and leukemic B and T cells: correlation with in vivo therapeutic responses to Campath-1H. Leuk Res 1998 Feb 22:2 185-91
CD55 (791Tgp72)	Spendlove, I, L. Li, J. Carmichael, & L. G. Durrant. Decay accelerating factor (CD55): A target for cancer vaccine? (1999) Cancer Research 59: 2282-2286.
CD59	Jarvis GA, Li J, Hakulinen J, Brady KA, Nordling S, Dahiya R, Meri S. Expression and function of the complement membrane attack complex inhibitor protectin (CD59) in human prostate cancer. Int J Cancer 1997 Jun 11 71:6 1049-55
CDC27	Wang RF, Wang X, Atwood AC, Topalian SL, Rosenberg SA. Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. Science 1999 May 21 284:5418 1351-4
CDK4	Wölfel, T., Hauer, M., Schneider, J., Serrano, M, Wölfel, C., Klehmann-Hieb, E., de Plaen, E., Hankeln, T., Meyer zum Büschenfelde, K, and Beach, D. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science 1995 Sep 1 269:5228 1281-4
CEA	Kass E, Schlom J, Thompson J, Guadagni F, Graziano P, Greiner JW. Induction of protective host immunity to carcinoembryonic antigen (CEA), a self-antigen in CEA transgenic mice, by immunizing with a recombinant vaccinia-CEA virus. Cancer Res 1999 Feb 1 59:3 676-83
c-myc	Watson PH, Pon RT, Shiu RP. Inhibition of c-myc expres-

Antigen	Reference
	sion by phosphorothioate antisense oligonucleotide identifies a critical role for c-myc in the growth of human breast cancer. Cancer Res 1991 Aug 1 51:15 3996-4000
Cox-2	Tsujii M et al, Cycloxygenase regulates angiogenesis induced by colon cancer cells. Cell 1998; 93:705-716
DCC	Gotley DC, Reeder JA, Fawcett J, Walsh MD, Bates P, Simmons DL, Antalis TM. The deleted in colon cancer (DCC) gene is consistently expressed in colorectal cancers and metastases. Oncogene 1996 Aug 15 13:4 787-95
DcR3	Genomic ampliphication of a decoy receptor for Fas ligand in lung and colon cancer. Pitti R et al, Nature 396, 699-703. 1998.
E6 / E7	Steller MA, Zou Z, Schiller JT, Baserga R. Transformation by human papillomavirus 16 E6 and E7: role of the insulin-like growth factor 1 receptor. Cancer Res 1996 Nov 1 56:21 5087-91
EGFR	Yang XD, Jia XC, Corvalan JR, Wang P, Davis CG, Jakobovits A: Eradication of established tumors by a fully human monoclonal antibody to the epidermal growth factor receptor without concomitant chemotherapy. Cancer Res 1999, 59(6):1236-43.
EMBP	Clinical study on estramustine binding protein (EMBP) in human prostate. Shiina H, Igawa M, Ishibe T. Prostate 1996 Sep 29:3 169-76.
Ena78	D.A. Arenberg et. al., Epithelial-neutrophil activating peptide (ENA-78) is an important angiogenic factor in non-small cell lung cancer. J. Clin. Invest. (1998) 102; 465-472.
farsyl transferase	
FGF8b and FGF8a	Dorkin TJ, Robinson MC, Marsh C, Bjartell A, Neal DE, Leung HY. FGF8 over-expression in prostate cancer is associated with decreased patient survival and persists in androgen independent disease. Oncogene 1999 Apr 29 18:17 2755-61
FLK-1/KDR	T. Annie T. Fong et al, SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vacularization and growth of multiple tumor type. Cancer Res, 59, 99-106, 1999
Folic Acid Receptor	Dixon KH, Mulligan T, Chung KN, Elwood PC, Cowan KH. Effects of folate receptor expression following stable transfection into wild type and methotrexate transport-

Antigen	Reference
	deficient ZR-75-1 human breast cancer cells. J Biol Chem 1992 Nov 25 267:33 24140-7
G250	Divgi CR, Bander NH, Scott AM, O'Donoghue JA, Sgouros G, Welt S, Finn RD, Morrissey F, Capitelli P, Williams JM, Deland D, Nakhre A, Oosterwijk E, Gulec S, Graham MC, Larson SM, Old LJ. Phase I/II radioimmunotherapy trial with iodine-131-labeled monoclonal antibody G250 in metastatic renal cell carcinoma. Clin Cancer Res 1998 Nov 4:11 2729-39
GAGE-Family	De Backer O, 10 others, Boon T, van der Bruggen P. Characterization of the GAGE genes that are expressed in various human cancers and in normal testis. Cancer Res 1999 Jul 1;59(13):3157-3165.
gastrin 17	Watson SA, Michaeli D, Grimes S, Morris TM, Crosbee D, Wilkinson M, Robinson G, Robertson JF, Steele RJ, Hardcastle JD. Anti-gastrin antibodies raised by gastrimmune inhibit growth of the human colorectal tumour AP5. Int J Cancer 1995 Apr 10 61:2 233-40
Gastrin-releasing hormone (Bombesin)	Wang QJ, Knezetic JA, Schally AV, Pour PM, Adrian TE. Bombesin may stimulate proliferation of human pancreatic cancer cells through an autocrine pathway. Int J Cancer 1996 Nov 15 68:4 528-34
GD2 / GD3 / GM2	Wiesner DA, Sweeley CC. Circulating gangliosides of breast-cancer patients. Int J Cancer 1995 Jan 27 60:3 294-9
GnRH	Bahk JY, Hyun JS, Lee H, Kim MO, Cho GJ, Lee BH, Choi WS. Expression of gonadotropin-releasing hormone (GnRH) and GnRH receptor mRNA in prostate cancer cells and effect of GnRH on the proliferation of prostate cancer cells. Urol Res 1998 26:4 259-64
GnTV	Hengstler JG, Arand M, Herrero ME, Oesch F. Polymorphisms of N-acetyltransferases, glutathione S-transferases, microsomal epoxide hydrolase and sulfotransferases: influence on cancer susceptibility. Recent Results Cancer Res 1998 154: 47-85
GP1	
gp100 / Pmel 17	Wagner SN, Wagner C, Schultewolter T, Goos M. Analysis of Pmel17/gp100 expression in primary human tissue specimens: implications for melanoma immuno- and gene-therapy. Cancer Immunol Immunother 1997 Jun 44:4 239-47
gp-100-in4	Kirkin AF, Dzhandzhugazyan K, Zeuthen J. Melanoma-associated antigens recognized by cytotoxic T lymphocytes. APMIS 1998 Jul 106:7 665-79

Antigen	Reference
gp15	Maeurer MJ, et al. New treatment options for patients with melanoma: review of melanoma-derived T-cell epitope-based peptide vaccines. Melanoma Res. 1996 Feb;6(1):11-24.
gp75 / TRP-1	Lewis JJ, Houghton AN. Definition of tumor antigens suitable for vaccine construction. Semin Cancer Biol 1995 Dec 6:6 321-7
hCG	Hoermann R, Gerbes AL, Spoettl G, Jüüngst D, Mann K. Immunoreactive human chorionic gonadotropin and its free beta subunit in serum and ascites of patients with malignant tumors. Cancer Res 1992 Mar 15 52:6 1520-4
Heparanase	Vlodavsky I, Friedmann Y, Elkin M, Aingorn H, Atzmon R, Ishai-Michaeli R, Bitan M, Pappo O, Peretz T, Michal I, Spector L, Pecker I. Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis [see comments]. Nat Med 1999 Jul 5:7 793-802
Her2 / neu	Lewis JJ, Houghton AN. Definition of tumor antigens suitable for vaccine construction. Semin Cancer Biol 1995 Dec 6:6 321-7
HMTV	Kahl LP, Carroll AR, Rhodes P, Wood J, Read NG. An evaluation of the putative human mammary tumour retrovirus associated with peripheral blood monocytes. Br J Cancer 1991 Apr 63:4 534-40
Hsp70	Jaattela M, et al. Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. EMBO J. 1998 Nov 2;17(21):6124-34.
hTERT (telomerase)	Vonderheide RH, Hahn WC, Schultze JL, Nadler LM. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. Immunity June;10: 673-679. 1999.
IGFR1	M.J. Ellis et. al., Insulin-like growth factors in human breast cancer. Breast Cancer Res. Treat. (1998) 52; 175-184.
IL-13R	Murata T, Obiri NI, Debinski W, Puri RK. Structure of IL-13 receptor: analysis of subunit composition in cancer and immune cells. Biochem Biophys Res Commun 1997 Sep 8 238:1 90-4
iNOS	Klotz T, Bloch W, Volberg C, Engelmann U, Addicks K. Selective expression of inducible nitric oxide synthase in human prostate carcinoma. Cancer 1998 May 15 82:10 1897-903
Ki 67	Gerdes, J., U. Schwab, H. Lemke, and H. Stein. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell prolifera-

Antigen	Reference
	tion. Int J Cancer 31, 13-20, 1983
KIAA0205	Guéguen M, Patard JJ, Gaugler B, Brasseur F, Renauld JC, Van Cangh PJ, Boon T, Van den Eynde BJ. An antigen recognized by autologous CTLs on a human bladder carcinoma. J Immunol 1998 Jun 15 160:12 6188-94
K-ras, H-ras, N-ras	Abrams SI, Hand PH, Tsang KY, Schlom J. Mutant ras epitopes as targets for cancer vaccines. Semin Oncol 1996 Feb 23:1 118-34
KSA (CO17-1A)	Zhang S, Zhang HS, Reuter VE, Slovin SF, Scher HI, Livingston PO. Expression of potential target antigens for immunotherapy on primary and metastatic prostate cancers. Clin Cancer Res 1998 Feb 4:2 295-302
LDLR-FUT	Caruso MG, Osella AR, Notarnicola M, Berloco P, Leo S, Bonfiglio C, Di Leo A. Prognostic value of low density lipoprotein receptor expression in colorectal carcinoma. Oncol Rep 1998 Jul-Aug 5:4 927-30
MAGE Family (MAGE1, MAGE3)	Marchand M, et al., Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. Int J Cancer 1999 Jan 18;80(2):219-30.
Mammaglobin	Watson MA, Dintzis S, Darrow CM, Voss LE, DiPersio J, Jensen R, Fleming TP, Mammaglobin expression in primary, metastatic, and occult breast cancer. Cancer Res 1999 Jul 1 59:13 3028-31.
MAP17	Kocher O, Cheresh P, Lee SW. Identification and partial characterization of a novel membrane-associated protein (MAP17) up-regulated in human carcinomas and modulating cell replication and tumor growth. Am J Pathol 1996 Aug 149:2 493-500
Melan-A / MART-1	Lewis JJ, Houghton AN. Definition of tumor antigens suitable for vaccine construction. Semin Cancer Biol 1995 Dec 6:6 321-7
mesothelin	Chang K, et al. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. Proc Natl Acad Sci U S A. 1996 Jan 9;93(1):136-40
MIC A/B	Groh V, Steinle A, Bauer S, and Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. Science 1998, 279: 1737-1740.
MT-MMP's, such as MMP2, MMP3, MMP7, and MMP9	Sato H and Seiki M., Membrane-type matrix metalloproteinases (MT-MMPs) in tumormetastasis., J Biochem (Tokyo) 1996 Feb;119(2):209-15
Mox1	Candia AF, Hu J, Crosby J, Lalley PA, Noden D, Nadeau JH, Wright CV. Mox-1 and Mox-2 define a novel homeobox

Antigen	Reference
	gene subfamily and are differentially expressed during early mesodermal patterning in mouse embryos. Development 1992 Dec 116:4 1123-36
Mucin, such as MUC-1, MUC-2, MUC-3, and MUC-4	Lewis JJ, Houghton AN. Definition of tumor antigens suitable for vaccine construction. Semin Cancer Biol 1995 Dec 6:6 321-7
MUM-1	Kirkin AF, Dzhandzhugazyan K, Zeuthen J. Melanoma-associated antigens recognized by cytotoxic T lymphocytes. APMIS 1998 Jul 106:7 665-79
NY-ESO-1	Jager, E. et. al. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. J. Exp. Med. 1998, 187:265-270.
Osteonectin	Graham JD, Balleine RL, Milliken JS, Bilous AM, Clarke CL. Expression of osteonectin mRNA in human breast tumours is inversely correlated with oestrogen receptor content. Eur J Cancer 1997 Sep 33:10 1654-60
p15	Yoshida S, Todoroki T, Ichikawa Y, Hanai S, Suzuki H, Hori M, Fukao K, Miwa M, Uchida K. Mutations of p16Ink4/CDKN2 and p15Ink4B/MTS2 genes in biliary tract cancers. Cancer Res 1995 Jul 1 55:13 2756-60
P170 / MDR1	Trock BJ, Leonessa F, Clarke R. Multidrug resistance in breast cancer: a meta-analysis of MDR1/gp170 expression and its possible functional significance. J Natl Cancer Inst 1997 Jul 2 89:13 917-31
p53	Roth J, Dittmer D, Rea D, Tartaglia J, Paoletti E, Levine AJ. p53 as a target for cancer vaccines: recombinant canarypox virus vectors expressing p53 protect mice against lethal tumor cell challenge. Proc Natl Acad Sci U S A 1996 May 14 93:10 4781-6.
p97 / melanotransferrin	Furukawa KS, Furukawa K, Real FX, Old LJ, Lloyd KO. A unique antigenic epitope of human melanoma is carried on the common melanoma glycoprotein gp95/p97. J Exp Med 1989 Feb 1 169:2 585-90
PAI-1	Grøndahl-Hansen J, Christensen IJ, Rosenquist C, Brünner N, Mouridsen HT, Danø K, Blichert-Toft M. High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. Cancer Res 1993 Jun 1 53:11 2513-21
PDGF	Vassbotn FS, Andersson M, Westermark B, Heldin CH, Ostman A. Reversion of autocrine transformation by a dominant negative platelet-derived growth factor mu-

Antigen	Reference
	tant. Mol Cell Biol 1993 Jul 13:7 4066-76
Plasminogen (uPA)	Naitoh H, Eguchi Y, Ueyama H, Kodama M, Hattori T. Localization of urokinase-type plasminogen activator, plasminogen activator inhibitor-1, 2 and plasminogen in colon cancer. Jpn J Cancer Res 1995 Jan 86:1 48-56
PRAME	Kirkin AF, Dzhandzhugazyan K, Zeuthen J. Melanoma-associated antigens recognized by cytotoxic T lympho-cytes. APMIS 1998 Jul 106:7 665-79
Probasin	Matuo Y, Nishi N, Muguruma Y, Yoshitake Y, Kurata N, Wada F. Localization of prostatic basic protein (''probasin'') in the rat prostates by use of monoclo-nal antibody. Biochem Biophys Res Commun 1985 Jul 16 130:1 293-300
Progenipoietin	
PSA	Sanda MG, Smith DC, Charles LG, Hwang C, Pienta KJ, Schlom J, Milenic D, Panicali D, Montie JE. Recombinant vaccinia-PSA (PROSTVAC) can induce a prostate-specific immune response in androgen-modulated human prostate cancer. Urology 1999 Feb 53:2 260-6.
PSM	Kawakami M, Nakayama J. Enhanced expression of prostate-specific membrane antigen gene in prostate cancer as revealed by in situ hybridization. Cancer Res 1997 Jun 15 57:12 2321-4
RAGE-1	Gaugler B, 7 others, Van den Eynde BJ. A new gene coding for an antigen recognized by autologous cytoly-tic T lymphocytes on a human renal carcinoma. Immunogenetics 1996;44(5):323-330.
Rb	Dosaka-Akita H, Hu SX, Fujino M, Harada M, Kinoshita I, Xu HJ, Kuzumaki N, Kawakami Y, Benedict WF. Altered retinoblastoma protein expression in nonsmall cell lung cancer: its synergistic effects with altered ras and p53 protein status on prognosis. Cancer 1997 Apr 1 79:7 1329-37
RCAS1	Sonoda K, Nakashima M, Kaku T, Kamura T, Nakano H, Watanabe T. A novel tumor-associated antigen expressed in human uterine and ovarian carcinomas. Cancer 1996 Apr 15;77(8):1501-1509.
SART-1	Kikuchi M, Nakao M, Inoue Y, Matsunaga K, Shichijo S, Yamana H, Itoh K. Identification of a SART-1-derived peptide capable of inducing HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes. Int J Cancer 1999 May 5 81:3 459-66
SSX gene family	Gure AO, Tüüreci O, Sahin U, Tsang S, Scanlan MJ, Jäger E, Knuth A, Pfreundschuh M, Old LJ, Chen YT. SSX: a

Antigen	Reference
	multigene family with several members transcribed in normal testis and human cancer. Int J Cancer 1997 Sep 17 72:6 965-71
STAT3	Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr. Stat3 as an oncogene. Cell 1999 Aug 6;98(3):295-303
STn (mucin assoc.)	Sandmaier BM, Oparin DV, Holmberg LA, Reddish MA, MacLean GD, Longenecker BM. Evidence of a cellular immune response against sialyl-Tn in breast and ovarian cancer patients after high-dose chemotherapy, stem cell rescue, and immunization with Theratope STn-KLH cancer vaccine. J Immunother 1999 Jan 22:1 54-66
TAG-72	Kuroki M, Fernsten PD, Wunderlich D, Colcher D, Simpson JF, Poole DJ, Schlom J. Serological mapping of the TAG-72 tumor-associated antigen using 19 distinct monoclonal antibodies. Cancer Res 1990 Aug 15 50:16 4872-9
TGF-α	Imanishi K, Yamaguchi K, Suzuki M, Honda S, Yanaihara N, Abe K. Production of transforming growth factor-alpha in human tumour cell lines. Br J Cancer 1989 May 59:5 761-5
TGF-β	Picon A, Gold LI, Wang J, Cohen A, Friedman E. A subset of metastatic human colon cancers expresses elevated levels of transforming growth factor betal. Cancer Epidemiol Biomarkers Prev 1998 Jun 7:6 497-504
Thymosin β 15	Bao, L., Loda, M., Janmey, P. A., Stewart, R., Anand-Apte, B., and Zetter, B. R. Thymosin beta 15: a novel regulator of tumor cell motility upregulated in metastatic prostate cancer. Nature Medicine. 2 (12), 1322-1328. 1996
TNF-α	Moradi MM, Carson LF, Weinberg B, Haney AF, Twiggs LB, Ramakrishnan S. Serum and ascitic fluid levels of interleukin-1, interleukin-6, and tumor necrosis factor-alpha in patients with ovarian epithelial cancer. Cancer 1993 Oct 15 72:8 2433-40
TPA	Maulard C, Toubert ME, Chretien Y, Delanian S, Dufour B, Housset M. Serum tissue polypeptide antigen (S-TPA) in bladder cancer as a tumor marker. A prospective study. Cancer 1994 Jan 15 73:2 394-8
TPI	Nishida Y, Sumi H, Mihara H. A thiol protease inhibitor released from cultured human malignant melanoma cells. Cancer Res 1984 Aug 44:8 3324-9
TRP-2	Parkhurst MR, Fitzgerald EB, Southwood S, Sette A, Rosenberg SA, Kawakami Y. Identification of a shared HLA-A*0201-restricted T-cell epitope from the melanoma

Antigen	Reference
	antigen tyrosinase-related protein 2 (TRP2). Cancer Res 1998 Nov 1 58:21 4895-901
Tyrosinase	Kirkin AF, Dzhandzhugazyan K, Zeuthen J. Melanoma-associated antigens recognized by cytotoxic T lymphocytes. APMIS 1998 Jul 106:7 665-79
VEGF	Hyodo I, Doi T, Endo H, Hosokawa Y, Nishikawa Y, Tanimizu M, Jinno K, Kotani Y. Clinical significance of plasma vascular endothelial growth factor in gastrointestinal cancer. Eur J Cancer 1998 Dec 34:13 2041-5
ZAG	Sanchez LM, Chirino AJ, Bjorkman Pj. 1999, Crystal structure of human ZAG, a fat-depleting factor related to MHC molecules.Science 19;283(5409):1914-9
p16INK4	Quelle DE, Ashmun RA, Hannon GJ, Rehberger PA, Trono D, Richter KH, Walker C, Beach D,Sherr CJ, Serrano M. Cloning and characterization of murine p16INK4a and p15INK4b genes. Oncogene 1995 Aug 17;11(4):635-45
Glutathione S-transferase	Hengstler JG, Arand M, Herrero ME, Oesch F. Polymorphisms of N-acetyltransferases, glutathione S-transferases, microsomal epoxide hydrolase and sulfotransferases: influence on cancer susceptibility. Recent Results Cancer Res 1998 154: 47-85

In the following, a number of specific tumour-associated
antigens will be discussed in detail.

Prostate-specific membrane antigen, PSM

In U.S.A., prostate cancer is the second leading cause of
5 cancer death (app. 40,000 per year), and 200,000 patients per
year are diagnosed (Boring 1993). Approximately 1 out of 11
men eventually will develop prostatic cancer. Furthermore,
approximately 40-60% of prostate cancer patients eventually
develop extraprostatic extension of the disease (Babaian
10 1994). The main strategy in the present invention is to use a
therapeutic vaccine as a supplementary therapy to prostatec-
tomy in order to eliminate residual tumour tissue and metasta-
ses.

Several pathologic conditions are located to the prostate
gland, including benign growth (BPH), infection (prostatitis)
and neoplasia (prostatic cancer).

The biological aggressiveness of prostatic cancer is variable.
5   In some patients the detected tumour remains a latent histolo-
gic tumour and never becomes clinically significant. In other
patients, the tumour progresses rapidly, metastasises and
kills the patient in a relatively short time period (2-5
years).

10 The current primary treatment of prostate cancer is prostatec-
tomy. However, due to the extensive spreading of prostate
cancer cells the majority of prostatic cancer patients are not
cured by local surgery. Patients with non-confined disease
eventually receive systemic androgen ablation therapy, but the
15 annual death rate from prostatic cancer has not declined at
all over the 50 years since androgen ablation became standard
therapy for metastatic disease.

PSM is a membrane protein which is highly specific for pro-
static tissues, benign as well as malignant, although expres-
20 sion of PSM has also been observed in other tissues such as
renal tissue and renal tumor, small intestine, brain and tumor
neovasculature. Therefore, if surgery was successful,
prostatectomised cancer patients should theoretically express
PSM on residual malignant prostate tumour tissue or metastases
25 originating from the tumour. By inducing a strong CTL response
and/or a strong polyclonal antibody response towards PSM, it
is expected that residual tumour tissue can be eliminated.

Interestingly, upregulation of PSM expression is seen follow-
ing androgen-deprivation therapy of prostate cancer patients
30 (Wright 1996). This would make a PSM-targeted treatment very

well-suited to follow the traditional androgen-deprivation
therapy.

PSM was first identified in 1987 as a result of generating a
monoclonal antibody, 7E11-C5.3, raised against an isolated
5   human prostatic cancer cell, LNCaP (Horoszewicz 1987). The
antibody recognised both normal and malignant prostatic epi-
thelium, and was used in 1993 to purify and determine the
amino acid sequence of the PSM protein and eventually clone
the gene (Israeli 1993).

10 PSM is a type II transmembrane glycoprotein with a molecular
weight of 84 kD as predicted from the nucleic acid sequence
whereas the glycosylated version has an observed molecular
weight of 100-120 kD. Sequencing of the gene encoding PSM
revealed a putative membrane spanning region in connection
15 with three cytosolic arginine anchor residues. The extracellu-
lar part of PSM constitute 707 of the total 750 amino acids of
the protein, whereas the cytoplasmic domain is predicted to be
19 amino acids long (Israeli 1993). PSM-specific mRNA has been
detected in prostate tumour tissue (Israeli 1994), indicating
20 that the tumour antigen is not an aberrantly glycosylated
protein which is the case with e.g. the Tn- or sTn-tumour
antigens.

The full length PSM cDNA has been transfected into and ex-
pressed in a PSM negative human prostate cancer cell line, PC-
25 3 (Kahn 1994). Furthermore, the full length (2.65 kilobases)
cDNA has been transcribed and translated in vitro (Kahn 1994).

It has recently been demonstrated that PSM possesses hydroly-
tic activity resembling that of the N-acetylated α-linked
acidic dipeptidase (NAALADase) - in fact it has been demon-
30 strated that the two proteins are identical. NAALADase is a
membrane-bound hydrolase of the nervous system, which

catabolises the neuropeptide N-acetylaspartyl glutamate (NAAG)
in order to affect the glutamatergic signalling processes. It
is still not known whether this activity of PSM has any rele-
vant biological function.

5 It is of some importance to predict whether undesired cross-
reactivity with other proteins accessible for CTLs or antibo-
dies would be expected following treatment with an autovaccine
inducing PSM-specific immune responses. It has been shown that
a part of the coding region of the PSM gene (amino acids
10 positions 418-567) has 54% homology to the human transferrin
receptor (Israeli 1993). Also, complete sequence identity with
the NAALADase enzyme has been found, cf. above. No identifica-
tion of a functionally relevant similarity with other known
peptidases could be made.

15 The homology to the transferrin receptor is very low and will
preferably be disrupted in some of the inventive constructs.
The observed sequence identity with human NAALADase is not
expected to be an obstacle for a PSM-vaccine, partly because
of the low ability of antibodies and CTLs to penetrate the
20 blood-brain barrier. Altogether, even with the most PSM-like
construct, it is not expected to experience prohibitive cross-
reactivity with other proteins in the patients.

From earlier studies it is clear that PSM is expressed on most
prostate cancer cells and prostate originating metastases
25 tested. Further, most other cancers tested, such as carcino-
mas, sarcomas and melanomas of different tissues as well as a
large panel of non-prostatic human cancer cell lines have
proven PSM negative.

In addition to this, a very large number of other tissues have
30 been found to be PSM negative. These include colon, breast,
lung, ovary, liver, urinary bladder, uterus, bronchus, spleen,

pancreas, tongue, esophagus, stomach, thyroid, parathyroid, adrenal, lymph node, aorta, vena cava, skin, mammary gland and placenta. However, RT-PCR has revealed the existence of PSM mRNA in some of these tissues.

5 Although PSM is predominantly found as a membrane bound mole-cule on prostate tissue small amounts of PSM can also be detected in the sera of normal individuals and in elevated levels in prostate cancer patients (Rochon 1994, Murphy 1995). The level of circulating PSM in these patients therefore
10 allows a serological monitoring of the effectiveness of a PSM vaccine.

In conclusion, based on the entire amount of data available to date, PSM is an antigen with a high specificity for human prostate tissue and tumours originating therefrom. This means
15 that in patients who have undergone prostatectomy, PSM is a tumour quasi-specific self-antigen. An effective PSM vaccine is therefore likely to target mainly prostatic or prostate-originating metastatic tissue.

As will be clear from Example 1 the method of the invention
20 preferably entails that foreign T_H-cell epitope is introduced in a part of the PSM amino acid sequence defined by SEQ ID NO: 2 positions 16-52 and/or 87-108 and/or 210-230 and/or 269-289 and/or 298-324 and/or 442-465 and/or 488-514 and/or 598-630 and/or 643-662 and/or 672-699. Furthermore, a modified PSM
25 molecule which has a foreign T_H-epitope introduced in these positions is also a part of the invention.

Accordingly, the invention also pertains to an analogue of human PSM which is immunogenic in humans, said analogue com-prising a substantial part of all known and predicted CTL and
30 B-cell epitopes of PSM and including at least one foreign T_H epitope as discussed herein. Preferred PSM analogues are those

wherein the at least one foreign T_H epitope is present as an
insertion in the PSM amino acid sequence or as a substitution
of part of the PSM amino acid sequence or as the result of
deletion of part of the PSM amino acid sequence, and most
5 preferred analogues are those wherein a foreign T_H-cell epitope
is introduced in a part of the PSM amino acid sequence defined
by SEQ ID NO: 2 positions 16-52 and/or 87-108 and/or 210-230
and/or 269-289 and/or 298-324 and/or 442-465 and/or 488-514
and/or 598-630 and/or 643-662 and/or 672-699.

10 *Human Chorionic Gonadotropin (HCG)*

The relationship between embryonic markers and malignant
phenotypes has been under discussion for many decades. An
increasing body of data suggests that at least one such mar-
ker, human chorionic gonadotropin beta (hCGβ), is consistently
15 detected on cancer cells of many different histological ori-
gins, and that expression of this protein often correlates
with increased metastatic properties. A humoral immune re-
sponse directed against this soluble protein may reduce the
chances of tumour spreading and/or may inhibit the recurrence
20 of new primary growths post-surgery.

Human chorionic gonadotropin belongs to a family of glycopro-
tein hormones, including follicle-stimulating hormone (FSH),
thyroid-stimulating hormone (TSH), and luteinizing hormone
(LH), all of which are important regulators of reproductive
25 expression and fetal survival. The members of this family of
hormones are heterodimers, which share a common α-chain. The
β-chain is unique to each hormone and provides the specifi-
city, with the β-chain of LH exhibiting the strongest sequence
homology to hCGβ (approximately 80%). The apparent molecular
30 weight of hCG-holo, is 37 kD, of which one third is contri-
buted by carbohydrate. The post-translational sugar modifica-
tions include both N-linked and O-linked carbohydrate. Abun-

dant sialic acid residues are present and these give the
protein a large negative charge. The crystal structure of hCG-
holo has been solved (Lapthorn *et al.*, 1994).

Based on the crystal structure it was found that hCG exhibits
5 homology to a family of growth factors, including PDGF and
TGFβ (Lapthorn *et al.*, 1994). This suggests that hCG expres-
sion may help regulating cancer cell growth.

Human chorionic gonadotropin is a glycoprotein hormone, which
is produced by the placental syncytiotrophoblasts soon after
10 conception, and it is essential for successful gestation in
the pregnant woman.

The pathophysiological role of embryonic markers for the
development or maintenance of a cancer mass is not known.
However, it is of interest to note that trophoblasts (where
15 these proteins are normally produced) have both *angiogenic* and
invasive characteristics, both of which are also necessary
properties for a cancer cell. Further, it has been suggested
that hCG (or its subunits) can inhibit maternal cellular
immune responses to fetal tissue. For example, studies have
20 shown that hCG directly suppresses T cell responses (Jacoby *et
al.*, 1984) and it has been proposed that because the lymph
nodes draining (in this case) a primary melanoma tumour, are
immunosuppressed, a more favourable environment for metastatic
tumours to establish themselves may result. As a consequence,
25 expression of hCGβ may help cancer cells spread into the
secondary lymphoid organs. Finally, as mentioned above, struc-
tural homology between hCG and a number of growth factors have
been demonstrated. Another possibility is therefore that
secretion of hCG by cancer cells may give the tumour a growth
30 advantage.

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Expression of hCGβ has been shown in many different types of cancer, for example: a) Prostate adenocarcinoma: positivity for hCGβ on tissue sections were seen for patients with poor prognosis, irrespective of the histological grade of the

5 tumour (Sheaff et al., 1996), b) different kinds of lung carcinomas; squamous cell (SQCC), adenocarcinoma (AC), and large cell (LCC), all showed a high percentage of reactivity for hCGβ (Boucher et al. 1995), c) pancreatic adenocarcinoma (Syrigos et al., 1998), d) neuroblastomas, brain cancers,

10 retinoblastomas (Acevedo et al., 1997), e) malignant melanoma (Doi et al., 1996), f) bladder carcinomas (Lazar et al., 1995). A recent paper describes a DNA approach, in which mice were immunized with a hCGβ expression construct (Geissler et al., 1997). In this in vivo model inhibition of tumour growth

15 was strongly associated with CTL-activity, however high titers of antibodies (which neutralized the biological effect of intact hCG on its cellular receptor) were also detected.

The use of hCG as an immunogen has been described in several papers, focussing on its use as a contraceptive vaccine (Tal-

20 war et al., 1976 and Talwar et al., 1994). A very high degree of efficacy and safety has been observed in an anti-fertility clinical trial, using a vaccine against hCG-holo (Talwar et al., 1994). Phase I clinical trials of cancer patients with a vaccine against a synthetic carboxy-terminal peptide of hCGβ

25 conjugated to diphteria toxoid have also been conducted (Tri- ozzi et al., 1994) and phase II trials are underway. Despite the fact that the idea to use hCGβ as a cancer vaccine target has been around for some time, it has not been explored in conjunction with the AutoVac technology.

30 It is known that cells from non-embryonic tissue, or benign neoplasms, do not express hCGβ. Therefore, there should be no potential side effects from vaccination against this molecule (apart from the effects on pregnancy). Because it is expressed

by so many different kinds of cancers this molecule has been
proposed to be the "definitive cancer biomarker" (Acevedo *et
al.*, 1995 and Regelson W., 1995) and as such would be an
attractive target to go after.

5　Suitable animal models for Further studies of the efficacy of
a hCG based vaccine can be found in Acevedo *et al.*, Cancer
Det. and Prev. Suppl. (1987) 1: 477-486, and in Kellen *et al.*,
Cancer Immunol. Immun.Ther. (1982) 13: 2-4.

Her2

10　The tyrosine kinase receptors Her2 and EGFr are believed to
play a crucial role in the malignant transformation of normal
cells and in the continued growth of cancer cells. Overexpres-
sion is usually linked to a very poor prognosis. During the
past few years there has been many reports concerning the use
15　of antibodies against these receptors as therapy for cancers
that overexpress either or both of these receptors. Genentech
Inc. has finished several successful clinical trials on breast
cancer patients using a monoclonal antibody against Her2 and
has recently obtained an FDA approval for the marketing of the
20　anti-Her2 monoclonal antibody preparation, Herceptin®.

The autovacination technology disclosed herein as applied on
the Her2 molecule would elicit polyclonal antibodies that
would predominantly react with Her2. Such antibodies are
expected to attack and eliminate tumour cells as well as
25　prevent metastatic cells from developing into metastases. The
effector mechanism of this anti-tumour effect would be medi-
ated via complement and antibody dependent cellular cytotoxi-
city.

Dependent on the choice of constructs, the induced autoantibo-
30　dies could also inhibit cancer cell growth through inhibition

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of growth factor dependent oligo-dimerisation and internalisa-
tion of the receptors. And, most importantly, the Her2 ana-
logues are expected to be able to induce CTL responses di-
rected against known and/or predicted Her2 epitopes displayed
5   by the tumour cells

Her2 is a member of the epidermal growth factor receptor
family (c-erbB) which consists of four different receptors to
date: c-erbB-1 (EGFr), c-erbB-2 (Her2, c-Neu), c-erbB-3 and c-
erbB-4 (Salomon et al, 1995). C-erbB-3 and c-erbB-4 are less
10 well characterised than EGFr and Her2. Her2 is an integral
glycoprotein. The mature protein has a molecular weight of 185
kD with structural features that closely resembles the EGFr
receptor (Prigent et al, 1992). EGFr is also an integral
membrane receptor consisting of one subunit. It has an appa-
15 rent molecular weight of 170 kD and consists of a surface
ligand-binding domain of 621 amino acids, a single hydrophobic
transmembrane domain of 23 amino acids, and a highly conserved
cytoplasmic tyrosine kinase domain of 542 amino acids. The
protein is N-glycosylated (Prigent et al, 1994).

20 All proteins in this family are tyrosine kinases. Interaction
with the ligand leads to receptor dimerisation, which in-
creases the catalytic action of the tyrosine kinase (Bernard.
1995, Chantry 1995). The proteins within the family are able
to homo- and heterodimerise which is important for their
25 activity. The EGFr conveys growth promoting effects and stimu-
lates uptake of glucose and amino acids by cells (Prigent et
al 1992). Her2 also conveys growth promoting signals. Only
EGFr binds EGF and TGF-alpha. These ligands do not bind to the
other receptors in the family (Prigent et al., 1992). The
30 ligands for Her2 are not fully determined. However, heregulin
has been shown to induce phosphorylation by activating Her2.
This does not appear to be due to a direct binding to the
receptor but it is believed that heregulin is a ligand for

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erbB-3 and erbB-4 which then activates Her2 by oligo-dimerisa-
tion (*Solomon et al 1995*).

The homology between the proteins of EGF receptor family is
most pronounced in the tyrosine kinase domain at the cytoplas-
5 mic part of the molecules (82% between EGFr and Her2). The
homology is less in the extracellular part - from 41% to 46%
in different domains (*Prigent et al, 1992*).

The epidermal growth factor receptor is expressed on normal
tissues in low amounts, but it is overexpressed in many types
10 of cancers. EGFr is overexpressed in breast cancers (*Earp et
al, 1993, Eppenberger 1994*), gliomas (*Schlegel et al, 1994*),
gastric cancer (*Tkunaga et al, 1995*), cutaneous squamous
carcinoma (*Fujii 1995*), ovarian cancer (*van Dam et al, 1994*)
and others. Her2 is also expressed on few normal human tissues
15 in low amount, most characteristically on secretory epithelia.
Over expression of Her2 occurs in about 30% of breast, gas-
tric, pancreatic, bladder and ovarian cancers.

The expression of these receptors varies depending on the
degree of differentiation of the tumours and the cancer type,
20 e.g., in breast cancer, primary tumours overexpress both
receptors; whereas in gastric cancer, the overexpression
occurs at a later stage in metastatic tumours (*Salomon et al,
1995*). The number of overexpressed receptors on carcinoma
cells is greater than 10^6/cell for several head and neck can-
25 cers, vulva, breast and ovarian cancer lines isolated from
patients (*Dean et al, 1994*).

There are several reasons why the EGFr family of receptors
constitute suitable targets for tumour immunotherapy. First,
they are overexpressed in many types of cancers, which should
30 direct the immune response towards the tumour. Second, the
tumours often express or overexpress the ligands for this

family of receptors and some are hypersensitive to the proliferative effects mediated by the ligands. Third, patients with tumours that overexpress growth factor receptors often have a poor prognosis. The overexpression has been closely

5   linked with poor prognosis especially in breast cancer, lung cancer and bladder cancer (2) and is apparently associated with invasive/metastatic phenotypes, which are rather insensitive to conventional therapies (*Eccles et al, 1994*).

Overexpression of Her2 is in some cases a result of amplifica-

10 tion of the gene and in other cases increased transcription and translation. The overexpression of Her2 is associated with poor prognosis in breast, ovarian cancers, gastric cancer, bladder cancer and possibly in non-small cell lung cancers (*Solomon et al, 1995*).

15 Phase I clinical trials have been performed with a bispecific antibody in patients with advanced breast and ovarian cancer. The antibody was bispecific against Her2 and FcγRI (*Weiner et al, 1995*). Efficient lysis of Her2 over expressing tumour cells was observed with a bispecific antibody against Her2 and

20 CD3 (*Zhu et al, 1995*).

Treatment of scid mice xenografted with human gastric cancer with an anti-Her2 monoclonal antibody prolonged the survival of the mice (*Ohniski et al, 1995*). The anti-tumour activities of monoclonal antibodies against Her2, *in vitro* and *in vivo* is

25 not due to an identical mechanism; they may act as partial ligand agonists, alter Her2 receptor turnover and phosphorylation or may affect dimerization (*Lupu et al, 1995*).

Similarly, it has been shown that antibodies to EGFr can also interfere with growth factor interactions. (Baselga *et al*,

30 1994, Modjahedi *et al*, 1993a, Wu *et al*, 1995, Modjahedi *et al*,

1993b, Tosi *et al*, 1995, Dean *et al*, 1994, Bier *et al*, 1995, Modjtahedi *et al*, 1996, Valone 1995).

Hence, an important embodiment of the methods of the invention is one wherein the foreign T-cell epitope is introduced in a
5   part of the Her2 amino acid sequence defined by the amino acid numbering in SEQ ID NO: 3 positions 5-25 and/or 59-73 and/or 103-117 and/or 149-163 and/or 210-224 and/or 250-264 and/or 325-339 and/or 369-383 and/or 465-479 and/or 579-593 and/or 632-652 and/or 653-667 and/or 661-675 and/or 695-709 and/or
10 710-730, cf. the Examples.

Accordingly, the invention also relates to an analogue of human Her2 which is immunogenic in humans, said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of Her2 and including at least one foreign
15 T$_H$ epitope as discussed herein. It is preferred that the at least one foreign T$_H$ epitope is present as an insertion in the Her2 amino acid sequence or as a substitution of part of the Her2 amino acid sequence or as the result of deletion of part of the Her2 amino acid sequence. Most preferred analogues are
20 those defined above, *i.e.* those wherein the foreign T-cell epitope is introduced in a part of the Her2 amino acid sequence defined by SEQ ID NO: 3 positions positions 5-25 and/or 59-73 and/or 103-117 and/or 149-163 and/or 210-224 and/or 250-264 and/or 325-339 and/or 369-383 and/or 465-479 and/or 579-
25 593 and/or 632-652 and/or 653-667 and/or 661-675 and/or 695-709 and/or 710-730.

FGF8b

It has been shown by several investigators that FGF8b can induce proliferation, transformation, differentiation and in
30 some cases greatly increase the tumorigenicity of mammalian cells and tissues (Tanaka 1992, Kouhara 1994, Lorenzi 1995,

MacArthur 1995a, Crossley 1996a, 1996b, Ghosh 1996, Ohuchi 1997a, Rudra-Ganguly 1998). These effects are primarily mediated through the binding of FGF8b to members of the fibroblast growth factor receptors FGFR2, FGFR3, and FGFR4 (MacArthur

5   1995b, Blunt 1997, Tanaka 1998). Thus, cells expressing one of these receptors and FGF8(b) have been shown to provide an autocrine growth-signaling cascade leading to proliferation. The biological effect of FGF8b is most likely partly mediated through the JAK/STAT3 pathway, since we and others have ob-

10 served that addition of FGF8b to the growth medium of certain cells does promote phosphorylation of STAT3, a feature suspected to render cells resistant to apoptosis (Catlett-Falcone 1999).

In addition to the in vitro observations mentioned above, it
15 has recently been shown that FGF8(b) expression is significantly upregulated in both prostate and breast cancers (Marsh 1999, Dorkin 1999). We therefore believe, that an autovaccine against FGF8(b) will be a very efficient means of treating a number of FGF8-expressing tumors, or perhaps increase their
20 sensitivity towards apoptosis inducing agents.

Prostate cancer

The biological aggressiveness of prostatic cancer is variable. In some patients the detected tumor remains a latent histologic tumor and never becomes clinically significant. In other
25 patients, the tumor progresses rapidly, metastasizes, and kills the patient in a relatively short time (2-5 years).

For the purpose of diagnosis, and to follow the response to therapy of prostate cancer, determination of the circulating levels of two proteins has primarily been used: prostatic acid
30 phosphatase (PAP) and prostate specific antigen (PSA) (Nguyen 1990, Henttu 1989). Due to disrupture of the normal architec-

ture of the prostate gland in response to cancer development, these soluble proteins are released into the circulation where they can be detected as markers for e.g. metastatic spread.

The current primary treatment of prostate cancer is prostatec-
5 tomy. However, due to the extensive spreading of prostate cancer cells the majority of prostatic cancer patients are not cured by local surgery. Patients with non-confined disease receive systemic androgen ablation therapy, but the annual death rate from prostatic cancer has not declined at all over
10 the 50 years since androgen ablation became standard therapy for metastatic disease.

RT-PCR analysis has shown that FGF8 mRNA is produced by the human prostatic epithelial tumor cell lines LNCaP, PC-3, ALVA-31, and DU145 respectively, with FGF8b being the most
15 prominent isoform (Tanaka 1995, Ghosh 1996). The growth of the androgen-responsive LNCaP cells are stimulated by addition of recombinant FGF8b (Tanaka 1995), while DU145 cells could be growth inhibited by transfection with vira expressing anti-sense FGF8b (Rudra-Ganguly 1998). This, together with
20 evidence from developmental studies discussed below, indicate a role for FGF8b in maintaining the cancerous state of these cell lines.

Using FGF8a cDNA for in situ hybridization experiments, Leung and co-workers have shown that a high proportion (80% (n=106),
25 and 71% (n=31)) of prostatic cancers produce FGF8 mRNA, and that the amount of FGF8 mRNA correlate with the severeness of the tumors (P<0.0016, and P<0.05, respectively) (Leung 1996, Dorkin 1999). Using a monoclonal anti-FGF8b antibody, this isoform was shown responsible for the overexpression of FGF8b
30 (Dorkin 1999). Additionally, men with tumors which expressed high levels of FGF8 had worse survival (P=0,034), and that FGF8 expression persisted in androgen independent prostate

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cancers (Dorkin 1999). According to the data presented by
Dorkin and coworkers the expression of FGF8b in prostate
cancer could predict patient survival.

Immunohistochemical analysis using a monoclonal antibody
5 against FGF8, has detected the protein in 93% (n=43) of human
prostate cancers (Tanaka 1998). Normal prostatic tissue or
benign prostatic hyperplasia does produce low levels of FGF8
mRNA, and does not contain detectable amounts of FGF8 protein
(Leung 1996, Yoshimura 1996, Ghosh 1996, Tanaka 1998, Dorkin
10 1999).

These results indicate that an autovaccine against FGF8(b)
would be reactive against prostatic tumor tissue and thus,
extremely valuable in the treatment of prostatic cancer.

Breast cancer

15 The current treatment of breast cancer is surgery. However,
due to the extensive spreading of breast cancer cells a large
part of breast cancer patients are not cured by local surgery.
Patients with non-confined disease eventually receive androgen
ablation therapy, chemotherapy, and or radiation therapy. The
20 annual death rate from breast cancer is, however, still rela-
tively high.

FGF8 was originally isolated from a mouse mammary carcinoma
cell-line (SC-3), from which the expression could be induced
by adding androgen to the medium (Nonomura 1990). The protein
25 is also known to induce the proliferation of these as well as
other mammalian cells. Recently FGF8b mRNA has been shown to
be present in eight (n=8) human breast cancer cell lines
(MDA-MB-231, MDA-MB-415, ZR 75-1, T-47-D, SK-BR-III, PMC-42,
HBL-100 and MCF-7) (Tanaka 1995, Payson 1996, Wu 1997, Marsh
30 1998).

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Wnt-1 transgenic mice infected with mouse mammary tumor virus (MMTV) develop mammary tumors. FGF8 transcription is activated in 50% of these tumors (MacArthur 1995c, Kapoun 1997).

5 Transgenic mice that are carrying the FGF8b cDNA under control of the very specific mouse mammary tumor virus (MMTV) promoter, are shown to spontaneously develop FGF8b expressing mammary tumors (Coombes, personal communication).

Very recent data shows that FGF8(b) expression is upregulated in breast cancer (Tanaka 1998, Marsh 1999). Tanaka and co-
10 workers used a new monoclonal FGF8 antibody in immunohistochemical studies. They showed that FGF8 was present in 67% (n=12) of breast cancers, and that androgen receptors were present in 89% of FGF8 positive breast diseases (Hyperplasia, Fibroadenoma, Intraductal papilloma, and cancers),
15 which would allow the autocrine growth promoting loop to be involved in the progression of breast cancers (Tanaka 1998). Using a semi-quantitative RT-PCR method, it was shown that elevated levels of FGF8 mRNA were found in malignant compared to non-malignant breast tissues. Significantly more malignant
20 tissues were expressing FGF8 ($p=0.019$) at significantly higher levels ($p=0.031$) (68 breast cancers and 24 non-malignant breast tissues) (Marsh 1999).

It has not yet been fully established that FGF8(b) functions as an autocrine growth factor. However, the fact that a large
25 number of tumors overexpress FGF8b argues strongly that an autovaccine against FGF8b could be effective against a large percentage of breast and prostate cancers. The data reported by Marsh, Dorkin, and Tanaka indicate that an autovaccine against FGF8(b) could be used for treatment of both breast and
30 prostate cancers, and the rather vague data presented by Dorkin et. al, is a further support of the opinion that FGF8 is involved in the proliferation of human cancer cells.

Description of FGF8b

FGF8 belongs to the family of fibroblast growth factors
(FGFs). These growth regulatory proteins are small ~200 amino
acid residue proteins that all are involved in the induction
5 of proliferation and differentiation of a wide range of cells.
For a recent review of the involvement of the fibroblast
growth factors in vertebrate limb development, see Johnson
1997. The FGF family members are evolutionary related and
share 20-50% amino acid sequence identity.

10 FGF8b is a splice variant of FGF8, originally termed androgen
induced growth factor (AIGF). AIGF was first identified as a
protein secreted by a murine mammary carcinoma derived cell
line (SC-3) upon stimulation with androgen (Nonomura 1990).
The murine FGF8 gene contains 6 exons, potentially coding for
15 eight different FGF8-isoforms (FGF8a-h), differing only in the
N-terminal part of the molecules (Crossley 1995, MacArthur
1995b). Human FGF8 has the same gene structure as the murine
gene. However, due to a stop codon in exon 1B, human FGF8 can
possibly exist in four different isoforms namely FGF8a, FGF8b,
20 FGF8e, and FGF8f (Gemel 1996). The gene structures and the
amino acid sequences of the four human isoforms are illu-
strated in Fig. 5.

Mature FGF8b contains 193 amino acid residues, and has a
calculated molecular weight of 22.5kDa. The highly basic
25 protein contains 21 arginine and 14 lysine residues resulting
in a calculated isoelectric point of 10.84, and a calculated
positive charge of 19,8 at pH 7.0. It contains two cysteine
residues, and has two potential N-glycosylation sites. Due to
the nature of the investigations performed involving FGF8b
30 very little is known about the FGF8b protein moiety. It has,
however, been expressed heterologously from bacteria, purified
by the use of a C-terminal hexa-Histidine tag, and *in vitro*

refolded to a soluble and biologically active state (MacArthur
1995a, Blunt 1997).

Biological activity of FGF8b

As mentioned above, FGF8(b) was first isolated as a factor
5   that was released from a androgen dependent mouse mammary
tumor cell line, and it has been shown that this protein can
induce the proliferation of these cells. The morphological
changes mimic those induced by testosterone, which is also
know to induce the synthesis of FGF8(b) mRNA (Tanaka 1992).
10   The proliferation can be inhibited by FGF8(b) antisense oligos
(Nonomura 1990, Tanaka 1992, and Yamanishi 1994). Indeed, a
human prostate cancer cell line DU145 could be growth inhi-
bited by transfection with vira expressing anti-sense FGF8b
(Rudra-Ganguly 1998). Recent data shows that FGF8b induces
15   phosphorylation of STAT3 - a protein that is suspected to be
involved in resistance to apoptosis (Catlett-Falcone 1999,
Johnston, C.L., unpublished results).

FGF8b has by several investigators been shown very efficient
in inducing the transformation of NIH3T3 or SC115 cells (Miya-
20   shita 1994, Kouhara 1994, Lorenzi 1995, MacArthur 1995a). By
using recombinantly expressed proteins, it has also been shown
that this induction of morphological changes is far more
efficient with FGF8b than when using FGF8a or FGF8c (MacArthur
1995a, Ghosh 1996). Interestingly, the N-terminal half of the
25   FGF8b molecule alone, was shown to be sufficient for transfor-
mation of NIH3T3 cells, and even the small FGF8b specific
peptide (QVTVQSSPNFT) could enable the cells to grow 2-3 times
longer than normal in 0.1% serum (Rudra-Ganguly 1998). Fur-
thermore, NIH3T3 cells stably transfected with an expression
30   vector encoding FGF8b has been reported to be very tumorigenic
when injected intraocularly into nude mice (Kouhara 1994,
Ghosh 1996).

In vivo, FGF8b is known to be expressed at certain stages of development in vertebrates. A summary of the biological roles assigned to FGF8(b) is shown in Table 1. For reviews on the involvement of FGF8 in vertebrate development see Goldfarb
5 1999, and Johnson 1997.

Table: Various sites/tissues known to produce FGF8, and the proposed biological role(s).

Action/mechanism/presence (species)	References
Present in the developing limb buds (mouse)	Heikinheimo 1994, Ohuchi 1994
Limb bud outgrowth (chicken)	Kuwana 1997, Xu 1998
Induction of ectopic limb formation from mesoderm (chicken)	Crossley 1996b
Induction of midbrain formation from the caudal diencephalon (chicken)	Crossley 1996a
Initiation of wing outgrowth in a wingless mutant (chicken)	Ohuchi 1997a
Role in dorsoventral patterning of the gastrula (zebrafish)	Fürthauer 1997
Required during gastrulation, cardiac, craniofacial, forebrain, midbrain and cerebellar development (tissue specific knockout mice)	Meyers 1998
Role in tooth morphogenesis (mouse)	Kettunen 1998

It is believed that FGF8(b) mediates its action through bind-
25 ing to the fibroblast growth factor receptors (FGFR's). Specifically, FGF8b is known to be able to activate FGFR2c, FGFR3c, FGFR4c, and to some extent also FGFR1c, but not FGFR1b, -2b or -3b (MacArthur 1995b, Blunt 1997). In case of the induction of outgrowth of ectopic chicken limbs, it is
30 implicated that FGF10, FGFR2, and FGF8 interact and that this could be sufficient for outgrowth (Kuwana 1997, Xu 1998).

These results support the hypothesis that FGF8(b) can act in
an auto- and paracrine manner, leading to the normal outgrowth
and patterning of several anatomical structures during verte-
brate development. Importantly, FGF8 "total knock out" mice do
5 not survive most likely due to the elaborate involvement of
the protein in the development of the embryo.

Homology to other proteins

It is of significant importance to predict whether undesired
cross-reactivity with other proteins accessible for antibodies
10 would be expected following treatment with an autovaccine
inducing FGF8b specific autoantibodies. Due to the high degree
of sequence identity between FGF8b and the other FGF8 mole-
cules, an autovaccine will be expected to cross-react with
these proteins. This, however, will presumably be advanta-
15 geous, since none of these proteins are reported to be ex-
pressed in tissues or by cell-lines that do not already ex-
press FGF8b.

Amino acid residues 55 through 175 of FGF8b shows a relatively
low but significant degree of sequence identity to the other
20 FGFs. It is commonly accepted (and several times proven) that
a significant degree of sequence identity between two protein
domains is also reflected in a high degree of tertiary struc-
ture similarity. Therefore, the FGF family members are all
generally expected to be structurally similar. The three
25 dimensional structure of FGF2 has been resolved from crystals
as well as in solution (Ago 1991, Zhang 1991, Zhu 1991, Eriks-
son 1993, Blaber 1996, Moy 1996). FGF2 is composed entirely of
beta-sheet structure, comprising a three-fold repeat of a
four-stranded antiparallel beta-meander. This beta-barrel
30 structure is totally conserved between interleukin 1, FGF2 (or
basic FGF), and FGF1 (or acidic FGF). Nuclear magnetic reso-
nance analysis of FGF2 in solution has shown that the N-termi-

nal part of the molecule forms a relatively flexible struc-
ture. The remaining part of FGF8b (amino acid residues 1-54
and 176-215) only shows a low degree of sequence identity to
known proteins.

5 Based on the structural and alignment data, it is generally
assumed that the three dimensional structural core of the
other fibroblast growth factors closely resemble those of FGF1
and FGF2. These structural considerations are important fac-
tors in our design of the FGF8b mutant autovaccine molecules.
10
Importantly, due to the relatively low degree of sequence
identity between FGF8 and any of the other members of the FGF
family, the surface of FGF8 would be very different from that
of other FGFs, thereby minimizing the cross-reactivity of
15 FGF8b autovaccine generated antibodies with other FGF family
members. Due to the very low degree of homology to other
proteins than the fibroblast growth factors, we do not expect
an autovaccine against FGF8b to cross-react with any other
proteins.

20 It should be emphasized, however, that an autovaccine against
FGF8b probably would cross react with all isoforms of FGF8.
This will, however, presumably not be a problem since none of
the FGF8 isoforms are expected to be expressed at significant
levels in the adult. It is even possible that this cross
25 reaction will be beneficial in the treatment of cancer, since
it has been shown that at least some cancer cell lines express
other isoforms in addition to FGF8b.

Tissue distribution of FGF8b

Ideally, the induced autoantibodies and the subsequent effec-
30 tor mechanisms as well as the expected CTL response raised by
autovaccination should only be directed towards tissues that

are to be eliminated in the patient. Therefore, the tissue distribution of the antigen, which is targeted by an autovaccine, is an issue of great importance concerning the safety of the vaccine.

5   Table: Expression of FGF8b in various tissues and cells

Human

Breast cancer cell lines (MDA-MB-231, MDA-MB-415, ZR 75-1, T-47-D, SK-BR-III, PMC-42, HBL-100, and MCF-7)	((RT-PCR) Tanaka 1995, Payson 1996, Wu 1997, Marsh 1999)
Breast tumors	((mAb) Tanaka 1998, (RT-PCR)Marsh 1999)
Normal breast tissue	((RT-PCR)Wu 1997, Marsh 1999 (mAb) Tanaka 1998)
Prostate cancer (93%)	((in situ hyb.) Leung 1996, Dorkin 1999, (mAb) Tanaka 1998)
Breast disease	((mAb) Tanaka 1998)
Prostatic tumor cells (LNCaP, PC-3, DU145, and ALVA-31)	((RT-PCR) Tanaka 1995, Ghosh 1996, Schmitt 1996)
fetal kidney	((Northern blot) Ghosh 1996)
adult prostate, testis, kidney, neurons	((RT-PCR) Ghosh 1996, Wu 1997, Dorkin 1999)
teratocarcinoma cells (Tera-2)	((RT-PCR) Wu 1997)

Murine

Breast cancer cell lines (SC-115, RENCA)	((RT-PCR) Yoshimura 1996)
Hypothalamus, Testis	((RT-PCR) Yoshimura 1996)
Mammary tumors (Wnt-1 transgenic)	((Northern blot) MacArthur 1995c)
Embryonic brain	((in situ hyb.) Crossley 1995, Heikinheimo 1994, Ohuchi 1994, Shimamura 1997, (RT-PCR) Blunt 1997)
Ovary, testis	((Northern blot) Valve 1997)
Developing face and limb buds	((pAb) MacArthur 1995b (in situ hyb.) Heikinheimo 1994, Ohuchi 1994, Crossley 1995)
Gastrula	((in situ hyb.) Crossley 1995)

Chicken

Embryonic brain	((in situ hyb.) Crossley 1996a)

developing limb buds   ((in situ hyb.) Ohuchi
                                  1997a,b)
Rat
Prostate and testis   (RT-PCR) Scmitt 1996

The above table shows a wide selection of tissue distribution,
5   and cell line data of FGF8b expression. As seen from the
table, most of the data regarding tissue distribution is
generated using the sensitive RT-PCR method. This is because
Northern blotting analysis does not detect any FGF8b mRNA in
any normal tissues except from fetal kidney. From this scarce
10   data, it is generally assumed that expression of FGF8b mRNA in
the adult is very limited, and thus, an autovaccine against
FGF8b would presumably not be reactive against normal tissue.
Due to the fact that small amounts of FGF8b could interact in
unknown systems in the adult, the tissue distribution of the
15   protein needs further analysis. There are, however, in our
opinion no indications that an autovaccine against FGF8b would
result in serious unwanted effects on the patients.

Effects of antibodies against FGF8b

So far, no attempts to treat prostate cancer using monoclonal
20   antibodies have been published. Clinical trials with monoclo-
nal antibodies are ongoing in breast cancer therapy studies,
however.

Antibodies against FGF8b will probably block the interaction
between FGF8b and its receptors, which will inhibit the cell
25   membrane ruffling and cell proliferation, very likely
decreasing the motility and invasiveness of the cancer cells.

Hence, the invention also relates to embodiments of the
methods described herein where, where the foreign T-cell
epitope is introduced in a part of the FGF8b amino acid se-

quence defined by SEQ ID NO: 6 positions 1-54 and/or 178-215
and/or 55-58 and/or 63-68 and/or 72-76 and/or 85-91 and/or
95-102 and/or 106-111 and/or 115-120 and/or 128-134 and/or
138-144 and/or 149-154 and/or 158-162 and/or 173-177. It
5 should be noted that it is especially preferred *not* to intro-
duce variations or modifications in positions 26-45 and in the
C-terminus starting at amino acids 186-215, since these
stretches show the least homology with a recently discovered
protein, FGF-18, which seems to be expressed in a variety of
10 non-tumour tissues.

Accordingly, the invention also pertains to an analogue of
human/murine FGF8b which is immunogenic in humans, said ana-
logue comprising a substantial part of all known and predicted
CTL and B-cell epitopes of FGF8b and including at least one
15 foreign T_H epitope as discussed herein. It is preferred that
the at least one foreign T_H epitope is present as an insertion
in the FGF8b amino acid sequence or as a substitution of part
of the FGF8b amino acid sequence or as the result of deletion
of part of the FGF8b amino acid sequence. Most preferred
20 analogues in this embodiment are those where the foreign T-
cell epitope is introduced in a part of the FGF8b amino acid
sequence defined by SEQ ID NO: 6 positions 1-54 and/or 178-215
and/or 55-58 and/or 63-68 and/or 72-76 and/or 85-91 and/or
95-102 and/or 106-111 and/or 115-120 and/or 128-134 and/or
25 138-144 and/or 149-154 and/or 158-162 and/or 173-177.

Mucins

The invention also pertains to methods of the invention em-
ploying specifically modified versions of the human mucins,
especially any of MUC-1 through MUC-4, preferably MUC-1. The
30 analogues comprise the following structure

$$TR(-S-P-S-(TR)_m)_n$$
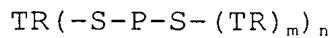
-where TR is a tandem repeat derived from the naturally occur-
ring mucin, P is a foreign T_H-epitope as discussed herein, S is
an inert spacer peptide having from 0 to 15 amino acid resi-
dues, preferably between 0 and 10 amino acid residues, and n
5   is an integer of from 1 to 30, and m is an integer from 1 to
10, preferably from 3 to 5.

When producing such a mucin analogue in e.g. a human cell line
or by purification from a tissue, the direct result will
normally not have a glycosylation pattern as desired, *i.e.* an
10 aberrant glycosylation pattern resembling that of a tumour
derived mucin. However, it is possible to produce the analogue
recombinantly in e.g. *E. coli* or by synthetic means, and
subsequently glycosylating the product enzymatically so as to
achieve a Tn or S-Tn glycosylation pattern specific for MUC-1
15 expressed on tumours. Alternatively, the polypeptide could be
prepared in a mammalian cell line or an insect cell line, eg.
Drosophila cells, which lacks the relevant enzyme or by ex-
pressing the protein intracellularly (by omitting a secretion
signal peptide) where glycosylation does not occur.

20 Nucleic acid fragments and vectors of the invention

It will be appreciated from the above disclosure that the
analogues can be prepared by means of recombinant gene tech-
nology but also by means of chemical synthesis or semisynthe-
sis; the latter two options are especially relevant when the
25 modification consists in coupling to protein carriers (such as
KLH, diphtheria toxoid, tetanus toxoid, and BSA) and non-
proteinaceous molecules such as carbohydrate polymers and of
course also when the modification comprises addition of side
chains or side groups to an polypeptide-derived peptide chain.

30 For the purpose of recombinant gene technology, and of course
also for the purpose of nucleic acid immunization, nucleic

acid fragments encoding the necessary epitopic regions and
analogues are important chemical products. Hence, an important
part of the invention pertains to a nucleic acid fragment
which encodes an analogue described above of any of the rele-
5 vant tumour-specific polypeptides, preferably a polypeptide
wherein has been introduced a foreign T_H-cell epitope by means
of insertion and/or addition, preferably by means of substitu-
tion and/or deletion. The nucleic acid fragments of the inven-
tion are either DNA or RNA fragments.

10 The nucleic acid fragments of the invention will normally be
inserted in suitable vectors to form cloning or expression
vectors carrying the nucleic acid fragments of the invention;
such novel vectors are also part of the invention. Details
concerning the construction of these vectors of the invention
15 will be discussed in context of transformed cells and microor-
ganisms below. The vectors can, depending on purpose and type
of application, be in the form of plasmids, phages, cosmids,
mini-chromosomes, or virus, but also naked DNA which is only
expressed transiently in certain cells is an important vector.
20 Preferred cloning and expression vectors of the invention are
capable of autonomous replication, thereby enabling high copy-
numbers for the purposes of high-level expression or high-
level replication for subsequent cloning.

The general outline of a vector of the invention comprises the
25 following features in the 5'→3' direction and in operable
linkage: a promoter for driving expression of the nucleic acid
fragment of the invention, optionally a nucleic acid sequence
encoding a leader peptide enabling secretion of or integration
into the membrane of the polypeptide fragment, the nucleic
30 acid fragment of the invention, and a nucleic acid sequence
encoding a terminator. When operating with expression vectors
in producer strains or cell-lines it is for the purposes of
genetic stability of the transformed cell preferred that the

vector when introduced into a host cell is integrated in the host cell genome. In contrast, when working with vectors to be used for effecting *in vivo* expression in an animal (i.e. when using the vector in DNA vaccination) it is for security rea-

5 sons preferred that the vector is not capable of being inte- grated in the host cell genome; typically, naked DNA or non- integrating viral vectors are used, the choices of which are well-known to the person skilled in the art.

The vectors of the invention are used to transform host cells

10 to produce the analogue of the invention. Such transformed cells, which are also part of the invention, can be cultured cells or cell lines used for propagation of the nucleic acid fragments and vectors of the invention, or used for recombi- nant production of the analogues of the invention. Alterna-

15 tively, the transformed cells can be suitable live vaccine strains wherein the nucleic acid fragment (one single or multiple copies) have been inserted so as to effect secretion or integration into the bacterial membrane or cell-wall of the analogue.

20 Preferred transformed cells of the invention are microorga- nisms such as bacteria (such as the species Escherichia [e.g. E.coli], Bacillus [e.g. Bacillus subtilis], Salmonella, or Mycobacterium [preferably non-pathogenic, e.g. M. bovis BCG]), yeasts (such as Saccharomyces cerevisiae), and protozoans.

25 Alternatively, the transformed cells are derived from a multicellular organism such as a fungus, an insect cell, a plant cell, or a mammalian cell. Most preferred are cells derived from a human being, cf. the discussion of cell lines and vectors below.

30 For the purposes of cloning and/or optimized expression it is preferred that the transformed cell is capable of replicating the nucleic acid fragment of the invention. Cells expressing

the nucleic fragment are preferred useful embodiments of the invention; they can be used for small-scale or large-scale preparation of the analogue or, in the case of non-pathogenic bacteria, as vaccine constituents in a live vaccine.

5   When producing the analogue of the invention by means of transformed cells, it is convenient, although far from essential, that the expression product is either exported out into the culture medium or carried on the surface of the transformed cell.

10 When an effective producer cell has been identified it is preferred, on the basis thereof, to establish a stable cell line which carries the vector of the invention and which expresses the nucleic acid fragment encoding the analogue. Preferably, this stable cell line secretes or carries the

15 analogue of the invention, thereby facilitating purification thereof.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with the hosts. The vector

20 ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., 1977). The pBR322 plasmid contains

25 genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the prokaryotic microorganism for expression.

30 Those promoters most commonly used in recombinant DNA construction include the B-lactamase (penicillinase) and lactose

promoter systems (Chang et al., 1978; Itakura et al., 1977;
Goeddel et al., 1979) and a tryptophan (trp) promoter system
(Goeddel et al., 1979; EP-A-0 036 776). While these are the
most commonly used, other microbial promoters have been dis-
5 covered and utilized, and details concerning their nucleotide
sequences have been published, enabling a skilled worker to
ligate them functionally with plasmid vectors (Siebwenlist et
al., 1980). Certain genes from prokaryotes may be expressed
efficiently in E. coli from their own promoter sequences,
10 precluding the need for addition of another promoter by arti-
ficial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast
cultures may also be used, and here the promoter should be
capable of driving expression. *Saccharomyces cerevisiase*, or
15 common baker's yeast is the most commonly used among eukaryo-
tic microorganisms, although a number of other strains are
commonly available such as *Pichia pastoris*. For expression in
Saccharomyces, the plasmid YRp7, for example, is commonly used
(Stinchcomb et al., 1979; Kingsman et al., 1979; Tschemper et
20 al., 1980). This plasmid already contains the trpl gene which
provides a selection marker for a mutant strain of yeast
lacking the ability to grow in tryptophan for example ATCC No.
44076 or PEP4-1 (Jones, 1977). The presence of the trpl lesion
as a characteristic of the yeast host cell genome then pro-
25 vides an effective environment for detecting transformation by
growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the
promoters for 3-phosphoglycerate kinase (Hitzman et al., 1980)
or other glycolytic enzymes (Hess et al., 1968; Holland et
30 al., 1978), such as enolase, glyceraldehyde-3-phosphate
dehydrogenase, hexokinase, pyruvate decarboxylase,
phosphofructokinase, glucose-6-phosphate isomerase, 3-
phosphoglycerate mutase, pyruvate kinase, triosephosphate

isomerase, phosphoglucose isomerase, and glucokinase. In
constructing suitable expression plasmids, the termination
sequences associated with these genes are also ligated into
the expression vector 3' of the sequence desired to be ex-
5   pressed to provide polyadenylation of the mRNA and termina-
tion.

Other promoters, which have the additional advantage of tran-
scription controlled by growth conditions are the promoter
region for alcohol dehydrogenase 2, isocytochrome C, acid
10 phosphatase, degradative enzymes associated with nitrogen
metabolism, and the aforementioned glyceraldehyde-3-phosphate
dehydrogenase, and enzymes responsible for maltose and
galactose utilization. Any plasmid vector containing a yeast-
compatible promoter, origin of replication and termination
15 sequences is suitable.

In addition to microorganisms, cultures of cells derived from
multicellular organisms may also be used as hosts. In prin-
ciple, any such cell culture is workable, whether from verte-
brate or invertebrate culture. However, interest has been
20 greatest in vertebrate cells, and propagation of vertebrate in
culture (tissue culture) has become a routine procedure in
recent years (Tissue Culture, 1973). Examples of such useful
host cell lines are VERO and HeLa cells, Chinese hamster ovary
(CHO) cell lines, and W138, BHK, COS-7 293 and MDCK cell
25 lines.

Expression vectors for such cells ordinarily include (if
necessary) an origin of replication, a promoter located in
front of the gene to be expressed, along with any necessary
ribosome binding sites, RNA splice sites, polyadenylation
30 site, and transcriptional terminator sequences.

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For use in mammalian cells, the control functions on the
expression vectors are often provided by viral material. For
example, commonly used promoters are derived from polyoma,
Adenovirus 2, and most frequently Simian Virus 40 (SV40). The
5 early and late promoters of SV40 virus are particularly useful
because both are obtained easily from the virus as a fragment
which also contains the SV40 viral origin of replication
(Fiers et al., 1978). Smaller or larger SV40 fragments may
also be used, provided there is included the approximately 250
10 bp sequence extending from the *Hind*III site toward the *Bgl*I
site located in the viral origin of replication. Further, it
is also possible, and often desirable, to utilize promoter or
control sequences normally associated with the desired gene
sequence, provided such control sequences are compatible with
15 the host cell systems.

An origin of replication may be provided either by construc-
tion of the vector to include an exogenous origin, such as may
be derived from SV40 or other viral (e.g., Polyoma, Adeno,
VSV, BPV) or may be provided by the host cell chromosomal
20 replication mechanism. If the vector is integrated into the
host cell chromosome, the latter is often sufficient.

Compositions of the invention

The invention also relates to an immunogenic composition which
comprises, as an effective immunogenic agent at least one of
25 the analogues described herein in admixture with a pharmaceu-
tically and immunologically acceptable carrier, vehicle,
diluent, or excipient, and optionally an adjuvant, cf also the
discussion of these entities in the description of the method
of the invention above.

30 Furthermore, the invention also relates to a composition
composition for inducing production of antibodies against any

one of the above discussed tumour antigens, the composition
comprising

- a nucleic acid fragment or a vector of the invention, and
- a pharmaceutically and immunologically acceptable diluent
5 and/or vehicle and/or carrier and/or excipient and/or
adjuvant.

Formulation and other specifics concerning such compositions
are discussed in the relevant section regarding nucleic acid
immunisation above.

10 EXAMPLE 1

Vaccination against PSM

In the following it will be described how a human autovaccine
against PSM can be developed through modification of the
molecule by insertion of one or more promiscuous foreign T
15 cell epitopes to reveal a panel of immunogenised PSM mole-
cules.

The constructs will be tested for their ability to induce
specific CTL responses against PSM bearing tumour cells.
Furthermore, the constructs will be tested for their ability
20 to induce antibodies which are cross-reactive with the native
parts of the PSM molecule. Subsequently, in several *in vitro*
assays and *in vivo* animal models the efficacy of the different
constructs will be evaluated. The induced antibodies will be
tested for their ability to activate complement via the clas-
25 sical pathway and to initiate ADCC via Fc-receptors. Finally,
the different molecules will be tested in animal models of
human prostate cancer.

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Strategy in designing a PSM autovaccine

Briefly, the PSM vaccination plan entails the following
experimental tasks

<u>Design and production of a panel of human PSM mutants</u>
5   -   Cloning of the PSM sequences from human and rat/mouse.
     -    Mutational work to generate immunogenized hPSM molecules.
     -    Expression of wild type and immunogenized hPSM molecules
          in *E. coli* and/or *Pichia pastoris* and/or mammalian cells
          and/or insect cells (such as the S_2 cell line).
10   -   Purification, refolding and characterization of the
          immunogenized hPSM molecules.

<u>DNA vaccination against PSM</u>
     -    Generation of hPSM DNA vaccination vectors encoding
          immunogenized hPSM molecules.
15   -   Testing of hPSM vaccination vectors in *in vitro* and *in
          vivo* experiments.
<u>Selection of hPSM candidates</u>
     -    Immunizations of mice/rabbits.
     -    ELISA.
20   -   FACS analysis.
     -    In case of antibody response: Tumor cell proliferative
          assay.
     -    T cell assays.

<u>Testing of the hPSM mutants *in vivo*</u>
25   -   Solid tumor/metastasis model in mice.

<u>Conceptual study: CTL induction by autovaccination</u>
     -    Construction of immunogenized mouse/rat PSM corresponding
          to the selected hPSM candidates (e.g. in the form of DNA
          vaccines).

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- Testing the immune response raised by mouse/rat PSM mu-
  tants in *in vitro* assays: Immunochemical assays, ELISA,
  FACS analysis, cellular assays, complement lysis of PSM
  bearing cells, ADCC assay, CTL activity assay, Tumor cell
5 proliferative assay, T cell presentation assays.
- Testing of the mPSM mutants *in vivo* in a solid tu-
  mor/metastasis model in mice.

Nomenclature of the hPSM constructs

PSM is a type II membrane protein of 750 amino acids, cf. SEQ
10 ID NO: 2 which sets forth the wild-type sequence with the
exception that Gly substitutes Trp in position 2 due to the
introduction of an *Nco*I site and a Kozak sequence in SEQ ID
NO: 1, where ggt substitutes tgg in positions 4-6. However,
native PSM (*i.e.* PSM having a Trp in position 2) has also been
15 used for some human PSM based autovaccine constructs.

In PSM, the extracellular part constitutes the 707 C-terminal
amino acids, whereas the cytoplasmic domain is predicted to be
19 amino acids long and the transmembrane part of the protein
consists of 24 amino acids (aa 20-43).

20 As starting point for the constructs, the splice variant PSM'
has also been used. Our version of this splice variant has the
amino acid sequence corresponding to residues 58-750 in SEQ ID
NO: 2. For ease of nomenclature, the regions in PSM' are
numbered according to the numbering in PSM (meaning that e.g.
25 region 2 consists of amino acids 87-108 in PSM and amino acids
30-51 in PSM'), cf. also the below discussion of the regions.

All the genetic constructs of human PSM are designated
hPSM__.__ (or hPSM'__.__ if PSM' is used as a starting point),
where the first __ is the insertion region used for insertion
30 of P2, and the second __ is the insertion region used for P30.

If P2 or P30 is not present in the protein, the number 0
(zero) is designated. The full length wild type hPSM is desig-
nated hPSM0.0 and the wild type hPSM lacking the cytoplasmic
and transmembrane parts is designated hPSM÷0.0. The 13 planned
5   immunogenized hPSM genes which all contain one P2 epitope and
one P30 epitope will be named hPSM1.1, hPSM6.1, hPSM8.1,
hPSM10.1, hPSM1.6, hPSM1.8, hPSM1.10, hPSM1.2, hPSM1.3,
hPSM1.5, hPSM2.1, hPSM3.1, hPSM10.3, hPSM6.3, hPSM'10.3,
hPSM'6.3, hPSM8.3, hPSM'8.3, and hPSM5.1, cf. details below.

10   In hPSM1.1, both the P2 and the P30 epitopes are inserted in
tandem in insertion region no. 1 (the membrane spanning re-
gion). Theoretically, this mutant, hPSM1.1, can be considered
a very attractive vaccine candidate for induction of antibody
production, because the whole extracellular domain of this
15   molecule is intact. For induction of CTL responses using
nucleic acid immunization, constructs such as hPSM10.3 and
hPSM6.3 are considered useful.

In order to facilitate the cloning and mutagenesis procedures,
much of the molecular construction work is done using either
20   the N-terminal (amino acids 1-436) or the C-terminal (amino
acids 437-750) part of the hPSM gene as starting material.
These two parts of the hPSM gene are designated hPSMI__.__ and
hPSMII__.__, respectively, where the first __ is the insertion
region used for insertion of P2, and the second __ is the
25   insertion region used for P30. Again, if P2 or P30 is not
present in the protein, the number 0 (zero) is designated, and
the wild types are named hPSMI0.0 and hPSMII0.0, respectively.
A special variant of hPSMI0.0 without the cytoplasmic part of
hPSM is designated hPSMI÷0.0.

30   Practically, most mutagenesis work is being done using
hPSMI0.0 and hPSMII0.0 as starting material.

The expressed hPSM mutant proteins will be designated PROS__.__, where the first __ is the insertion region used for insertion of P2, and the second __ is the insertion region used for P30. If P2 or P30 is not present in the protein, the

5   number 0 (zero) is designated. The wild type hPSM is designated PROS0.0. PROSII0.0 is the hPSM amino acids 437-750 protein product. HIS tagged proteins are called HIS-PROS__.__. As His tags has been used SEQ ID NO: 21 for expression in yeast and bacteria, whereas SEQ ID NO: 23 has been used for

10   expression in mammalian cells.

Cloning of the human PSM sequence

The LNCaP cell line which originates from a metastatic lesion of human prostatic adenocarcinoma was purchased from the American Type Culture Collection (ATCC). mRNA was isolated

15   from this cell line and reverse transcribed using a standard kit in order to obtain cDNA encoding the human PSM sequence. Using different sets of hPSM specific primers, PCR products encoding PSM(437-750) was obtained and further cloned into pUc19 (plasmid number pMR300) and verified by DNA sequencing.

20   This C-terminal part of wild type PSM is designated hPSM partII (hPSMII0.0).

Similarly, the wild type hPSM partI (hPSMI0.0) has been cloned into pUc19 using primers for amplifying partI both with (hPSMI0.0) and without (hPSMI÷0.0) the transmembrane+cytoplas-

25   mic domains. The clones were control sequenced and hPSMI0.0 and hPSMII0.0 were fused using ligation at the EcoRI site. The resulting clones of hPSM0.0 (SEQ ID NO: 2) and hPSM÷0.0 have been subcloned into a number of pro- and eucaryotic expression vectors and again sequence verified. The intracellularly

30   expressed form of human PSM (designated hPSM' - amino acids 58-750 of SEQ ID NO: 2) has also been constructed using the cDNA as starting material. This sequence has also been sub-

cloned into mammalian expression vectors and has been used as
starting material for some hPSM autovaccine constructs, e.g.
hPSM'10.3 and hPSM'6.3.

Cloning of the rat and mouse PSM sequences

5   Two EST (expressed sequence tag) clones containing murine PSM
    cDNA sequences (from fetal murine kidney and murine macropha-
    ges, respectively) were purchased from American Type Culture
    Collection (ATCC). Together, these EST's covered the mouse PSM
    cDNA sequence, and thus both full length mouse PSM (SEQ ID NO:
10 7 and 8) as well as murine PSM' (SEQ ID NO: 9 and 10) were
    subcloned into bacterial vectors and mammalian expression
    vectors. Murine PSM AutoVac construcs have also been made by
    insertion of P30 into the mouse PSM cDNA.

Expression of wild type hPSM in *E. coli*

15 The C-terminal part (amino acids 437-750) of hPSM, hPSMII0.0,
    has been cloned into the bacterial expression vector pET28b in
    order to obtain a product with an N-terminal poly-histidine
    (HIS) tail which facilitates easy large scale purification and
    identification with anti-poly-HIS antibodies. The protein
20 product of poly-HIS tagged hPSMII0.0 (protein product desig-
    nated HIS-PROSII0.0) was expressed in *E. coli*.

    The DNA encoding the truncated wild-type human PSM hPSM÷cyt0.0
    has also been expressed from pET28b in the *E. coli* strain
    BL21(DE3) where the expression product is located in inclusion
25 bodies. SDS-PAGE analysis of bacterial lysate showed a product
    with the expected migration and Western blotting with rabbit
    anti-HIS-PROSII0.0 also gave the expected band. Further, N-
    terminal sequencing of five amino acids of this product eluted
    from an SDS-PAGE gel gave the expected amino acid sequence.

The wild type hPSM constructs hPSM0.0, hPSM÷0.0 (as well as two hPSM mutants, hPSM1.1 and hPSM6.1, see below) have been cloned into different *E. coli* expression vectors in order to enable a more efficient expression and some degree of folding
5 of the recombinant proteins in *E. coli*. The chosen expression systems are:

pMCT6 which generates N-terminally His-tagged versions of the expressed recombinant proteins,

pGH433 which express the recombinant proteins in connection to
10 a 22 amino acid pelB leader sequence which should direct the protein to the periplasmic space of the *E. coli* bacteria, and

pMal-p2 in which recombinant proteins are expressed as C-terminal fusions to maltose binding protein (MBP) containing the natural MBP periplasmic leader sequence. Antibodies
15 against MBP can be used for detection of the fusion proteins and a carbohydrate coupled column can be used for affinity purification of the product.

However, E.coli expression experiments of the wild type hPSM proteins from these vectors only showed a fair expression
20 level from pMCT6. The problems of getting periplasmic expression of the wild type hPSM proteins are still not solved at present.

Expression of wild type hPSM in *Pichia pastoris*

Because of the relatively high molecular weight of the PSM
25 protein and its relatively high degree of glycosylation (app. 16% of the molecular weight) and in order to facilitate purification by elimination of the refolding step, it has been decided to implement alternative technology for eukaryotic expression of the recombinant proteins. Several well-charac-

terized eukaryotic expression systems have been evaluated, and for the initial screening of hPSM mutants, the yeast *Pichia pastoris* has been chosen as alternative to *E. coli* expression.

5 An expression system based on the yeast *Pichia pastoris* has been applied on PSM constructions. The glycosylation pattern of recombinant proteins expressed in this organism is expected to resemble the mammalian glycosylation patterns more than e.g. *Saccharomyces cerevisiae* due to a lesser branched mannosylation of the recombinant protein. It has been shown 10 that mannose receptor-mediated uptake of antigens by dendritic cells results in an approximately 100-fold more efficient presentation to T cells compared to fluid-phase endocytosed uptake. Therefore, mannosylation might play a role for the antigenicity (and especially the ability to induce CTL 15 responses) of the hPSM mutants and other antigens against which a CTL response is desirable.

A strain of *Pichia pastoris* as well as two different expression vectors have been purchased from Invitrogen. The vector pPICZαA carries a methanol inducible promoter upstream of the 20 polycloning site, whereas the pGAPZαA vector express proteins constitutively. Both vectors encode the α-factor secretion signal in order to export the recombinant proteins to the medium. The selection system of these vectors is zeocin resistance. The sequences encoding hPSM0.0, and hPSM÷0.0 (as well 25 as one hPSM mutant, hPSM1.1, cf. below) were subcloned into these vectors (in-frame with a C-terminal c-myc identification epitope, SEQ ID NO: 27).

Four *Pichia pastoris* strains (X-33, SMD1168, GS115, and KM71) differing e.g. in their growth requirements were transformed 30 with each of these (linearized) plasmids using electroporation. The transformation procedure was repeated several times with minor changes in order to obtain a large number of zeocin

resistant clones. Expression of wild type hPSM÷0.0 (as well as
hPSM1.1, see below) was obtained in the *Pichia pastoris* sys-
tem. The expressed products could be detected in Western
blotting of lysates of Pichia pastoris transformants both
5   using an anti-hPSM monoclonal antibody and an anti-c-myc
monoclonal antibody as primary. However, the recombinant
products were detected intracellularly.

Expression of wild type hPSM in mammalian cells

An expression system using CHO (<u>c</u>hinese <u>h</u>amster <u>o</u>vary) cells
10 will also be implemented for the final testing and production
of selected molecules.

So far, CHO cells have been transfected with wild type hPSM
and hPSM1.1 with/without in frame leader sequences in mamma-
lian expression vector pcDNA3.1. Geneticin resistant cells
15 have been obtained. In COS cells transiently transfected with
the same constructs, both hPSM0.0 and hPSM1.1 was detected in
Western Blotting of cell pellets using anti-hPSM monoclonal
antibody.

Tissue distribution of hPSM

20 A commercial kit has been purchased in order to determine
whether hPSM expression can be detected in various human
tissues including prostate, blood and brain. The method is
based on a dot blot detection of polyA containing mRNA ex-
tracted from 50 different human tissues. Preliminary results
25 do not indicate hyperexpression of hPSM in tissues such as
blood or brain. However, after the priority date of the pre-
sent application, others have demonstrated the presence of PSM
in other tissues, cf. above.

Design of the hPSM mutants

When designing the mutational work in PSM, some regions of the
protein are very important to preserve in the modified con-
structs, for example potential and identified T cell epitopes,
5 B cell epitopes and disulfide bridge cysteine residues. There-
fore, such "forbidden" regions have been identified within the
PSM sequence leaving a limited number of "open" regions of 20
amino acids or more available for exchange with the foreign T
helper epitopes P2 and/or P30. Per definition, the transmem-
10 brane region is also considered an "open" region since
autoantibodies directed against this region are irrelevant and
elimination of this sequence is believed to enhance the solu-
bility of the mutated PSM proteins but it cannot be excluded
that this region contain important CTL epitopes, hence the
15 preservation of the transmembrane region in e.g. hPSM10.3.

According to our expectation that the autovaccine will induce
a CTL response, it would be important to identify and preserve
potentially subdominant CTL epitopes in the constructs. Two
such epitopes are already known from the literature: 1) the
20 peptide comprising PSM amino acids 4-12 (LLHETDSAV) can be
presented on the human MHC class I molecule HLA-A2.1 (*Tjoa
1996*), and 2) the PSM(711-719) (ALFDIESKV) also binds HLA-A2.1
(*ref 25*). We have also searched the PSM amino acid sequence in
order to identify primary anchor residues of HLA class I
25 binding motifs as described by Rammensee et al. (*Rammensee,
1995*) for the most abundant HLA class I types (HLA-A1, HLA-A2,
HLA-A3, HLA-A23, HLA-A24 and HLA-A28), together constituting
80 % of the HLA-A types of the human population. Likewise,
potential HLA-B and HLA-C epitopes have been identified and
30 designated as "forbidden" areas.

Because the initial intention was to use C57/black x SJL F1
hybrid mice in case it was decided to use transgenic mice for

testing the PSM autovaccine constructs, certain potential
mouse H-2^b and H-2^s T helper epitopes have been identified and
considered "forbidden" regions (*Rammensee 1995*).

It is also important to preserve known antibody binding re-
5 gions in the PSM molecule, because they could be important in
the induction of specific anti-PSM autoantibodies. Five such
regions have already been described: PSM(63-68), PSM(132-137),
PSM(482-487) (WO 94/09820), PSM(716-723) and PSM(1-7) (Murphy,
1996). Using the computer based method of Hopp and Woods for
10 prediction of antigenic determinants, five regions are pre-
dicted: PSM(63-69), PSM(183-191), PSM(404-414), PSM(479-486)
and PSM(716-723) (Hopp 1983), some of these overlapping the
experimentally found B cell epitopes. These regions will also
be preserved in the PSM vaccine candidate molecules.

15 The PSM protein contains 4 cysteine residues (amino acid
positions 22, 196, 466 and 597) which will be preserved in the
immunogenized constructs because of their potential importance
in the formation of disulfide bridges.

Based on the above mentioned "forbidden" and "open" regions in
20 the hPSM protein, 10 regions suitable for insertion of foreign
T helper epitopes were identified:

Insertion regions in **hPSMI** (from initiation site to EcoRI
site, aa 1-437):

Region 1:   aa 16-52 in PSM (4 aa preceding TM, TM (24 aa) and
25   9 aa after TM = 37 aa)

Region 2:   aa 87-108 in PSM, aa 30-51 in PSM' (22 aa)

Region 3:   aa 210-230 in PSM, aa 153-173 in PSM' (21 aa)

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Region 4:   aa 269-289 in PSM, aa 212-232 in PSM' (21 aa)

Region 5:   aa 298-324 in PSM, aa 241-267 in PSM' (27 aa)

Insertion regions in **hPSMII** (from EcoRI site to termination site, aa 437-750):

5   Region 6:   aa 442-465 in PSM, aa 385-408 in PSM' (24 aa)

Region 7:   aa 488-514 in PSM, aa 431-457 in PSM' (27 aa)

Region 8:   aa 598-630 in PSM, aa 541-573 in PSM' (33 aa)

Region 9:   aa 643-662 in PSM, aa 586-605 in PSM' (20 aa)

Region 10:   aa 672-699 in PSM, aa 615-642 in PSM' (28 aa)

10 The insertion regions as well as the "forbidden" regions are represented graphically in Fig. 4.

A number of different immunogenized PSM constructs will be made by substitution of a segment of amino acids from two of the above listed insertion regions with P2 or P30. Each mutant
15 protein will thus contain both P2 and P30, although such constructions are only exemplary – single-mutants are also within the scope of the present invention. Experimentally, the mutations will be made in clones of hPSMI and hPSMII cDNA respectively, and the two mutated parts will subsequently be
20 combined by ligation (at the EcoRI site). The P2 and P30 epitopes have initially been inserted into insertion regions 1, 2, 3, 5, 6, 8 and 10 in order to create the mutants.

The sequences of P2 and P30 are:

P2: QYIKANSKFIGITEL (SEQ ID NO: 12, 15 aa), in this case
encoded by the nucleotide sequence cag tac atc aaa gct aac tcc
aaa ttc atc ggt atc acc **gag ct**g (SEQ ID NO: 11, 45 nucleo-
tides), where the sequence in boldface is a *Sac*I site. Other
5 codon choices may occur, depending on choice of cloning vector
and expression system

P30: FNNFTVSFWLRVPKVSASHLE (SEQ ID NO: 14, 21 aa), in this
case encoded by the nucleotide sequence ttc aac aac ttc acc
gt**a agc tt**c tgg ctg cgt gtt ccg aaa gtt <u>agc g**CT** **AGC** ca</u><u>**c ctg**</u>
10 <u>g</u>aa (SEQ ID NO: 13, 63 nucleotides), where boldface indicates
an *Hind*III site, single underlining indicates an *Eco*47III
site, capital letters indicates a *Bst*NI site, and double
underlining indicates an *Nhe*I site.

The following table summarizes the human PSM constructs used
15 herein:

Construct	P2 position in protein	P30 position in protein
hPSM0.0	÷	÷
hPSM÷0.0	÷	÷
hPSM'0.0	÷	÷
hPSM1.1	17-31	32-52
hPSM6.1	448-462	21-41
hPSM8.1	606-620	21-41
hPSM10.1	674-688	21-41
hPSM1.6	24-38	443-463
hPSM1.8	24-38	607-627
hPSM1.10	24-38	673-693
hPSM1.2	24-38	87-107
hPSM1.3	24-38	210-230
hPSM1.5	24-38	301-321
hPSM2.1	91-105	21-41
hPSM3.1	213-227	21-41
hPSM5.1	305-319	21-41

Construct	P2 position in protein	P30 position in protein
hPSM8.0	606-620	÷
hPSM10.0	674-688	÷
hPSM0.1	÷	21-41
hPSM1.0	24-38	÷
hPSM6.3	448-462	210-230
hPSM8.3	606-620	210-230
hPSM10.3	674-688	210-230
hPSM'6.3	391-405	153-173
hPSM'8.3	549-563	153-173
hPSM'10.3	617-631	153-173

Molecular constructions of the hPSM mutants

Mutations to insert P2 and P30 encoding sequences have been performed using both hPSMI0.0 and hPSMII0.0 as starting material.

In order to generate a majority of the hPSM mutants, P2 and P30 were initially inserted in hPSMI0.0 at insertion position 1. The resulting material (hPSMI1.0 and hPSMI0.1, respectively) was subsequently used as starting material for mutagenesis to insert P2 and P30 at positions 2,3 and 5 and for ligation with epitope mutated hPSMII. hPSMI1.0 was constructed using SOE (single overlap extension) PCR and subsequently sequence verified. hPSMI0.1 was constructed using the "Quick Change" technique and subsequently sequence verified.

The P2 epitope was inserted into positions 2, 3 and 5 of hPSMI1.0 using SOE-PCR to create hPSMI1.2, hPSMI1.3 and hPSMI1.5. These constructions were combined with hPSMII0.0 to create hPSM1.2, hPSM1.3 and hPSM1.5.

hPSMI2.1, hPSMI3.1 and hPSMI5.1 were constructed by SOE PCR using hPSMI0.1 as starting material. This material has been assembled with hSPMII0.0 by ligation at the EcoRI site in order to create the full length mutants hPSM2.1, hPSM3.1 and

5 hPSM5.1.

The P2 epitope was inserted at three different positions of hPSMII0.0 in order to create hPSMII6.0, hPSMII8.0 and hPSMII10.0 using the "Quick Change" technique, and these clones were subsequently sequence verified.

10 Subsequently, hPSMI0.1 was ligated with hPSMII6.0, hPSMII8.0 and hPSMII10.0 to obtain hPSM6.1, hPSM8.1 and hPSM10.1, and the clones were sequence verified.

Insertion of the P30 epitope in hPSMII is presently being done to generate hPSMII0.6, hPSMII0.8 and hPSMII0.10 using SOE PCR.

15 hPSM1.1 was constructed using two two-step PCR mutations followed by ligation in a *Hind*III site within the epitope sequence. The full length construct is sequence verified.

hPSM10.3, hPSM'10.3, and hPSM6.3 have been constructed using SOE-PCR. Several other hPSM variants with both P2 and P30

20 inserted in the extracellular part of hPSM are currently being constructed (hPSM'6.3, hPSM8.3, hPSM'8.3).

In addition to the originally contemplated mutants each con-taining both P2 and P30, four mutants which only contain a single foreign epitope have been constructed and sequence

25 verified: hPSM1.0, hPSM8.0, hPSM10.0 and hPSM0.1.

Expression of hPSM mutants in *E. coli*

In small-scale experiments, seven hPSM mutants, hPSM1.1,
hPSM6.1, hPSM8.1, hPSM10.1, hPSM2.1, hPSM3.1 and hPSM5.1 were
expressed from pET28b in the *E. coli* strain BL21(DE3), and
5   IPTG inducible products of the expected size were identified
on Coomassie Blue stained SDS-PAGE gels. However, a product of
hPSM1.1 was not detectable. The expression levels of these
hPSM mutants were very low compared to the product of the wild
type construct hPSM÷0.0. At this point, a fair expression
10 level of the hPSM mutants using the pET system in *E. coli*
seems impossible, and the use of other *E. coli* expression
systems and/or other host organisms is thus necessary.

As mentioned above, hPSM6.1 and hPSM1.1 have been subcloned
into different *E. coli* expression vectors in order to generate

15 -   N-terminally His-tagged versions of the expressed recombi-
         nant proteins using vector pMCT6,
     -    versions of the proteins expressed with the pelB leader
         sequence which directs the protein to the periplasmic space
         of the *E. coli* bacteria using vector pGH433, and
20 -   versions of the recombinant proteins expressed as a C-
         terminal fusion protein to maltose binding protein (MBP)
         using vector pMal-p2.

So far, a sufficient expression level from any of these con-
structs has not been obtained.

25 Since hPSM0.0 is fairly expressed in *E. coli* while a similar
expression level of full length hPSM0.0 or the hPSM mutants
has not been observed, it is possible that presence of the
cytoplasmic part of the hPSM molecule can somehow "inhibit"
the expression of the full-length hPSM constructs in *E. coli*.
30 To test this hypothesis, we initially made two genetic con-

structs of hPSM1.1 and hPSM6.1 without cytoplasmic domains. However, in *E.coli* expression experiments there were only weak expression of these ÷cyt gene products.

Expression of hPSM mutants in *Pichia pastoris*

5   In order to express the hPSM1.1 mutant protein from the yeast *Pichia pastoris*, the hPSM1.1 sequence has been subcloned (in-frame with a C-terminal c-myc identification epitope, SEQ ID NO: 27) into the two different expression vectors pPICZαA and pGAPZαA, and the sequences have been verified. hPSM1.1 expres-
10 sion (as well as hPSM÷0.0, see above) was detected intracellularly in the Pichia pastoris transformants.

Expression of hPSM mutants in mammalian cells

As mentioned above, hPSM1.1 has been subcloned into the mamma-lian expression vectors pcDNA3.1(+) and pZeoSV2 and these
15 constructs (and others) could be used for expression in e.g. CHO cells. Transient expression of hPSM1.1 as well as hPSM0.0 has been obtained in COS cells as verified by Western blot-ting.

DNA vaccination

20 DNA vaccination would, if effective, be very well suited for the PSM autovaccine – especially because this administration form has been shown to promote both CTL mediated immune reac-tions and antibody production. Therefore, it was the intention to perform a parallel study with the aim of investigating the
25 effect of DNA-vaccination of mice with appropriate vectors encoding immunogenized mouse ubiquitin and/or mouse TNFα. DNA vaccination with hPSM (and/or mutants) encoding naked DNA will also be done.

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Feasibility study using immunogenized ubiquitin for DNA vaccination

A feasibility study stating the effect of DNA vaccination with an immunogenized self protein was performed using ubiquitin
5 with an inserted T helper epitope from ovalbumin (UbiOVA) as a model protein. Sequences encoding UbiOVA as well as wild type ubiquitin were subcloned into the mammalian expression vector pcDNA3.1(-).

Groups of 5 BALB/c mice were immunized with 40 µg pcDNA-UbiOVA
10 or pcDNA-ubiquitin construct either intramuscularly in the quadriceps or intradermally. An control control group of received UbiOVA protein in complete Freunds adjuvant. Three and six weeks later, the immunizations were repeated with the only difference that the UbiOVA protein was emulsified in
15 incomplete Freunds adjuvant.

The mice were bled regularly and the anti-ubiquitin antibody titers were determined. In the DNA vaccinated UbiOVA groups, only very weak anti-ubiquitin antibody titers were obtained. Subsequently, all groups were boosted with UbiOVA protein in
20 incomplete Freunds adjuvant and bled in order to determine whether DNA vaccination with UbiOVA (and not ubiquitin) could potentiate the antibody response towards UbiOVA protein. The results of this experiment showed that there was no significant difference between the UbiOVA groups and the control
25 groups, all mice developed strong anti-ubiquitin antibodies upon this single UbiOVA/FIA boost.

DNA vaccination using hPSM constructs

Currently, various DNA vaccination experiments are ongoing using hPSM constructs. Various human PSM wildtype and AutoVac
30 constructs (such as e.g. hPSM0.0, hPSM÷0.0, hPSM'0.0, hPSM1.1,

hPSM10.3) have been subcloned into DNA vaccination vectors
(such as pcDNA3.1(+), pcDNA3.1(-), pVAX and pZeoSV2). In some
of the constructions, different leader sequences (such as the
CD11a, tPA, and IL-5 leader sequences; SEQ ID NOs: 29, 25, and
5 31, respectively) have been included directly N-terminally and
in-frame to allow secretion of the expressed hPSM proteins *in
vivo*. All the constructions in DNA vaccination vectors have
been verified by DNA sequencing and *in vitro* translation.

Mice of different strains (such as Balb/cA, Balb/cJ, DBA/2 and
10 A/J) have been injected with the above described hPSM DNA
vaccines either intradermally or intramuscularly and boosted
several times using the same constructs.

Serum samples have been obtained during the immunisation
period and stored at -20°C. These samples will be analysed for
15 presence of antibodies reactive with wild type hPSM.

Also, assays to monitor CTL and T helper proliferative re-
sponses in these mice are being established.

Preliminary results suggest that induction of both CTL as well
as antibody responses against PSM can be accomplished.

20 **Purification/characterization of HIS-tagged hPSM(437-750)
(HIS-PROSII0.0)**

HIS-tagged wild type hPSMII (HIS-PROSII0.0) was expressed from
pET28b, and solubilized inclusion bodies were applied to a gel
filtration FPLC column and eluded in a buffer containing 8 M
25 urea. Fractions predominantly containing hPSMII were subjected
to various refolding conditions to optimize the procedure.
Solubilized product dialyzed against a Tris buffer was esti-
mated to be more than 90 % pure using silver-stained SDS-PAGE.

Rabbits were immunized with the purified HIS-PROSII0.0 in
order to use the resulting antiserum for later detection and
possibly affinity purification of the hPSM mutants.

Purification/characterization of soluble hPSM (PROS÷0.0)

5 Wild type hPSM lacking the cytoplasmic and transmembrane
parts, PROS÷0.0, has been expressed in the *E. coli* strain
BL21(DE3) upon induction with IPTG and could be detected in
inclusion bodies. SDS-PAGE of this bacterial lysate followed
by Western blotting with rabbit anti-HIS-PROSII0.0 showed a
10 product with the expected migration. N-terminal sequencing of
the first five amino acids of this product eluted from an SDS-
PAGE gel showed the expected sequence corresponding to human
PSM. The product was subjected to a large series of
solubilization and refolding experiments. A product which stay
15 in solution can be obtained in a Tris buffer without denatu-
rant or reductant, but SDS-PAGE analysis reveals that the
material probably forms large aggregates. Mice and rabbits
have been immunized with this material in order to get anti-
body against hPSM e.g. for analytical purposes – the antisera
20 did not react with LNCap hPSM.

A batch of washed *E. coli* inclusion bodies of PROS÷0.0 has
been prepared for immunization of rabbits to generate a
polyclonal antiserum against PSM. Approximately 50% of the
protein content in the wet pelleted material contained was
25 PROS÷0.0. The antisera did not react with LNCap hPSM in Wes-
tern blotting.

Preparation of KLH-conjugated hPSM peptides for immunization

Three 15-mer peptides were synthesized in order to make an
immunogenic conjugate of known hPSM B cell epitopes with an
30 immunological carrier molecule to obtain a polyclonal anti-

serum which is able to recognize hPSM. These peptides contain the PSM B cell epitope plus 5-6 flanking PSM amino acids in each end.

The peptides were made by automatic synthesis, HPLC purified
5 and control-sequenced using Edman degradation.

A chemically linked conjugate was prepared by cross-linking the B cell epitope containing hPSM peptides KLH using a standard 1-step procedure with glutaraldehyde as the cross-linking
10 agent.

Synthesis of P2 and P30 peptides with flanking hPSM sequences

Six peptides have been designed which correspond to the P2 and P30 epitopes with 5 flanking hPSM amino acids in each end. The flanking amino acids correspond to the epitope insertion sites
15 6, 8 and 10. The peptides will be used in *e.g.* T cell proliferation assays, but also for other purposes such as ELISA or other *in vitro* assays. The peptide sequences are:

PSMpep007	P2 inserted in hPSM insertion position 6
	QERGV<u>QYIKANSKFIGITEL</u>RVDCT (SEQ ID NO: 15)
20 PSMpep008	P2 inserted in hPSM insertion position 8
	AVVLR<u>QYIKANSKFIGITEL</u>EMKTY (SEQ ID NO: 16)
PSMpep009	P2 inserted in hPSM insertion position 10
	MFLER<u>QYIKANSKFIGITEL</u>HVIYA (SEQ ID NO: 17)
PSMpep010	P30 inserted in hPSM insertion position 6
25	NSRLL<u>FNNFTVSFWLRVPKVSASHLE</u>VDCTP (SEQ ID NO: 18)
PSMpep011	P30 inserted in hPSM insertion position 8
	VVLRK<u>FNNFTVSFWLRVPKVSASHLE</u>SFDSL (SEQ ID NO: 19)

PSMpep012   P30 inserted in hPSM insertion position 10
                       LMFLE<u>FNNFTVSFWLRVPKVSASHLE</u>PSSHN (SEQ ID NO: 20)

The P2 and P30 epitopes are underlined. The peptides were made
by automatic synthesis and subjected to the process of HPLC
5 purification followed by control-sequencing using Edman degra-
dation.

Immunogenicity assays

Different experimental setups have been initiated in order to
produce materials and establish immunogenicity assays for the
10 future testing of and selection between the mutated PSM con-
structs.

**Generation of polyclonal rabbit anti-HIS-PROSII0.0 and anti-
KLH-PSM-peptide conjugate antisera**

15 Two rabbits were immunized with purified HIS-PROSII0.0, the
HIS-tagged C-terminal part of the hPSM protein (amino acids
437-750) emulsified 1:1 with complete Freunds adjuvant and
boosted twice (at days 28 and 55) with the same antigen emul-
sified in incomplete Freunds adjuvant.

20 Two rabbits were immunized with a cocktail consisting of the
KLH-PSM peptide conjugate plus each of the three free pep-
tides. These three peptides each contain a previously defined
B cell epitope of hPSM. The cocktail was emulsified 1:1 with
complete Freunds adjuvant. The rabbits were boosted twice (at
25 days 28 and 55) with the same antigen emulsified in incomplete
Freunds adjuvant.

Cross-reactivity between anti-HIS-PROSII0.0 and PSMpep005 and
cross-reactivity between anti-KLH-PSM peptide conjugate plus
peptides and HIS-PROSII0.0 was demonstrated in ELISA assays.

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The anti-HIS-PROSII0.0 antibody has the ability to recognize native hPSM in lysates of LNCaP cells in Western blotting.

Immunization of mice with retrovirally expressed hPSM0.0

At this stage of the PSM project, a serious obstacle is still
5 the lack of antibodies which are able to recognize correctly folded native hPSM. Therefore, an immunization experiment using retrovirally expressed hPSM0.0 was performed.

Six groups of Balb/c mice were immunized with either: 1) mitomycin C treated BALB/c fibrosarcoma cells (79.24.H8)
10 transduced with hPSM0.0 cDNA (CMV-Koz-hPSM), 2) mitomycin C treated BALB/c fibrosarcoma cells (79.24.H8), transduced with empty vector (CMVBipep), 3) packaging cells (BOSC) transfected with hPSM0.0 cDNA (CMV-Koz-hPSM), 4) packaging cells (BOSC) transfected with empty vector (CMVBipep), 5) retrovirus stock
15 expressing hPSM0.0 cDNA (CMV-Koz-hPSM) or 6) retrovirus stock, empty vector (CMVBipep).

At several time points, the mice were bled and the sera obtained tested for reactivity in ELISA for reactivity against HIS-PROSII0.0. Unfortunately, none of the mice developed
20 antibodies able to specifically recognize the HIS-PROSII0.0 preparation.

Establishment of an anti-hPSM ELISA

Purified HIS-PROSII0.0 was used for coating polystyrene micro-titre plates (Maxisorp) for the purpose of establishing an
25 ELISA assay for testing e.g. hybridoma supernatants or mouse and rabbit antisera. Sera from BALB/c mice immunized with the same preparation of HIS-PROSII0.0 were reactive with the immobilized HIS-PROSII0.0 at 0.5 µg per well using horse

radish peroxidase labelled rabbit anti-mouse Ig as secondary
antibody.

As mentioned above, the ability of an antiserum raised in
rabbits against KLH-PSMpep004-PSMpep005-PSMpep006 conjugate
5 mixed with the free peptides to react with immobilized HIS-
PROSII0.0 was demonstrated using this ELISA assay.

Using AquaBind® microtitre plates (cf. the disclosure in WO
94/03530 describing *i.a.* microtitre surfaces coated with
tresyl-activated dextran which are now marketed under the
10 registered trademark AquaBind), an ELISA using immobilized PSM
peptides (PSMpep004, PSMpep005 and PSMpep006) was established.
AquaBind® plates coated with these peptides could detect a
rabbit antiserum raised against the same preparation of anti-
gen. As mentioned above, rabbit anti-HIS-PROSII0.0 could be
15 detected on AquaBind® plates coated with PSMpep005.

**Establishment of an anti-hPSM Western blot using LNCaP cells
and monoclonal antibody 7E11C5**

7E11C5 B cell hybridomas which secrete mouse IgG2a monoclonal
antibody against an intracellular epitope of human PSM was
20 purchased from ATCC. Culture supernatant from approximately
90% dead cells was collected and used in Western blotting for
detection of human PSM in both membrane enriched preparations
of LNCaP cells as well as in LNCaP cell lysates. This antibody
was purified using protein G columns, and its reactivity with
25 LNCaP in Western blotting verified.

Establishment of a FACS method to detect hPSM on LNCaP cells

We have established to mutually independent FACS methods to
detect hPSM on LNCaP cells. Several problems are being ad-
dressed: The LNCaP cells grow very slow and in irregular

clumps, and therefore the method to prepare single cell sus-
pensions should be optimized. Secondly, the epitope recognized
by the mAb 7E11C5 is in the literature defined to be in the
cytoplasmic domain of hPSM. Therefore, the method to fix and
5   permeabilize the cells has been developed. For this purpose,
protein G purified 7E11C5 antibody has been FITC conjugated
and can thus be used without secondary antibody in FACS analy-
sis.

Also, a FACS method using the anti-hPSM monoclonal antibody
10 J591 which recognizes an epitope on the extracellular part of
hPSM, has been established. The antibody was obtained from BZL
Biologicals and FITC conjugated and subsequently used for FACS
analysis and sortings of e.g. LNCaP cells and hPSM transfec-
tants (see below).

15 **Establishing a cytotoxicity assay**

A method to purify dendritic cells from mouse bone marrow has
been implemented. Using model proteins, immunization of mice
with dendritic cells pulsed with model class I peptides and
protein has been optimized. Also, mice have been immunized
20 with a model protein (β-galactosidase) formulated in the form
of ISCOMS. T-cells purified from immunized mice have been *in
vitro* restimulated with different forms of the corresponding
antigens. The ability of these restimulated CTLs to lyse
different kinds of target cells (including pulsed dendritic
25 cells as well as transfectants expressing retrovirally ex-
pressed cytosolic class I peptide) was subsequently measured.
Two different *in vitro* assays measuring CTL activity have been
established, using either chromium release or and DNA fragmen-
tation (JAM method) as measures of cytotoxicity. Very nice
30 results were obtained with the β-galactosidase model protein
and with various combinations of MHC class I and class II
binding model peptides.

Establishment of tools to study breaking of autotolerance towards mouse PSM in mice.

It is the intention to study whether autotolerance to mouse PSM can be broken in mice by immunisation and/or DNA vaccina-
5 tion against murine PSM using murine PSM AutoVac molecules.

As mentioned above, cDNA encoding murine PSM (mPSM) has been obtained and DNA sequenced. Four mPSM variant molecules are being generated by insertion of P30 at well-defined sites in either full length mPSM or mPSM'. The constructs are as fol-
10 lows:

	mPSM amino acids substituted with P30	Length of molecule (no. of amino acids)
mPSM0.0	÷	752
mPSM'0.0	÷	694
mPSMX	255-271 (of SEQ ID NO: 8)	756
mPSMY	689-700 (of SEQ ID NO: 8)	760
mPSM'X	197-213 (of SEQ ID NO: 10)	698
mPSM'Y	631-642 (of SEQ ID NO: 10)	702

Initially, the mPSM wild type and analogue molecules are subcloned into DNA vaccination vectors and used for DNA vacci-
nation of mice.

20 It is the intention to analyse immune responses such as CTL responses and tumor elimination in the mice. For this, murine tumor cell lines will be transfected with wild type murine PSM (fused in-frame with an identification tag, e.g. the c-myc epitope, SEQ ID NO: 27, for detection purposes).

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in vivo PSM tumor models

Mouse T cell proliferation assays with P2 and P30

A series of T cell proliferation experiments has been con-
ducted in order to establish the T cell immunogenicity of P2
5   and P30 peptides in various mouse strains (BALB/cA (H-2^d),
C3H/Hen (H-2^k), DBA/1 (H-2^q) and C57BL/6 (H-2^b)). It is well
known that these epitopes are promiscuous in humans, but the T
cell promiscuity also needed to be confirmed in mice using
M&Es experimental setup. It was thus shown that P30 is T cell
10 immunogenic in the BALB/cA and C57BL/6 strains whereas neither
P2 or P30 were shown to be T cell immunogenic in the C3H/Hen
strain. In DBA/1, T cells could be raised against P2.

Generation of hPSM expressing mouse tumor cells

For the use of a hPSM specific tumor model in mice as well as
15 for the use in tumor cell proliferative assays, a panel of
hPSM expressing mouse tumor cells are being established.

One approach is to generate these cell lines by transducing
the murine tumor cell lines with retroviral vectors encoding
the full-length wild type hPSM0.0 cDNA.

20 Three different constructs encoding full length wild type cDNA
encoding human PSM inserted into the polycloning site of the
retroviral vector CMVBipep was constructed, two of these
containing a short Kozak sequence upstream of the start codon.

These constructs were transduced into three different mouse
25 tumor cell lines: P815 (mastocytoma, H-2^d), B16-F10 (melanoma,
H-2^b) and 79.24.H8 (fibrosarcoma, H-2^d) using the BOSC pack-
aging cell line. Geneticin resistant clones have been obtained
for all three cell types, and it was verified in PCR analysis

on genomic DNA template that the retroviral constructs were
integrated in the host cells. It has not yet been possible to
detect an expressed PSM gene product in Western blot or FACS
analysis using the 7E11C5 monoclonal antibody.

5 Two stable mouse tumor cell lines harboring membrane bound
wild type human PSM have been established by transfection.
This was done using hPSM0.0 cDNA subcloned in the mammalian
expression vector pcDNA3.1(÷) under the control of the CMV
promoter and containing a Kozak sequence upstream of the start
10 codon.

The resulting plasmid was transfected into two different mouse
tumor cell lines: 79.24.H8 (fibrosarcoma, Balb/c derived) and
SalN (fibrosarcoma, A/J derived). Geneticin resistant cultures
were obtained and subjected to Western blotting and FACS
15 analysis using the J591 and 7E11C5 anti-hPSM monoclonal anti-
bodies. Using the J591 antibody, the cells were FACS sorted
several rounds until a hPSM positive population was obtained.
hPSM expression was again verified by intracellular FACS
staining using the 7E11C5 antibody. It was also checked by
20 FACS analysis that the MHC class I expression levels were the
same level as the levels of the parental cell lines.

Cultures of 79.24.H8 and SalN cell lines expressing hPSM were
cloned by limiting dilution. Several clones were obtained and
tested for different hPSM expression levels by FACS analysis
25 using the anti-hPSM monoclonal antibody J591.

79.24.H8 cells expressing hPSM were transfected with the gene
encoding B7.1 for use in e.g. *in vitro* assays to monitor hPSM
specific CTL responses and/or interferon-gamma release. The
cells were FACS sorted one time using an anti-B7.1 antiserum.

Establishment of a hPSM specific tumor model in mice

It has been decided to establish at least two *in vivo* tumor
models in immune competent mice in order to determine the
anti-tumor effect of antibodies raised in mice against the

5 immunogenized hPSM molecules. This will hopefully be done by
injecting syngeneic mouse tumor cell lines modified to express
wild type hPSM on the surface membrane. Cells which form solid
tumors and/or cells which are known to metastasize will be
used. Cell lines which can be implanted in syngeneic mice

10 without being rejected due to the presence of the foreign hPSM
molecule will be used in the model. The ability of the hPSM
vaccines to eliminate such tumor cells will be used for the
selection of the hPSM vaccine candidates.

To evaluate the growth of Sa1N cells transfected with the full

15 length human PSM, different doses ($2x10^6$ and $5x10^6$) of the hPSM
transfected Sa1N cells (S-PSM, sorted 5 times) were injected
subcutaneously at the lower right flank of groups of A/J mice.
However, solid tumors did not establish. Subsequently, three
clones of S-PSM cells with different expression level of hPSM

20 were injected subcutaneously in 3 groups of A/J mice at a dose
of 10^7 cells/mouse. The sizes of the established tumors were
measured with a caliber measuring two different diameters
which were multiplied to give the tumor size in mm^2. These
values were compared for the three groups. Within 3-6 days,

25 all mice developed a solid tumor-like structure which dis-
appeared again approximately by day 15. This is likely to be
due to the presence of human PSM on the tumor cell surfaces,
although it has not yet been verified. Sa1N cells transfected
only with the pcDNA3.1 vector continued to grow as solid

30 tumors in mice.

A similar picture was observed in mice injected with 10^6,
$5x10^6$, or 10^7 79.24.H8 cells transfected with hPSM and sorted

several rounds for hPSM expression. These cells (termed 79-
PSM) also did not establish as tumors in Balb/c nor DBA/2
mice. However, when a clone of hPSM transfected 79.24.H8
cells, 79-PSM.3, was injected into Balb/c or DBA/2 mice, the
5 mice developed solid tumor-like structures which disappeared
again by day 10-20. Vector-transfected 79.24.H8 continued to
grow in Balb/c mice.

It still remains to be evaluated if these "tumors" are treat-
able, or if a better tumor model can be established based on
10 the described S-PSM and 79-PSM cell lines and clones.

Conclusions

In the molecular construction work we have succeeded in clo-
ning of the human PSM gene and obtaining the mouse PSM cDNA.
An array of fully sequenced immunogenized hPSM autovaccine
15 constructs have been produced. The hPSM mutants as well as
different wild type hPSM molecules have been expressed in *E.
coli*, and it was found and verified that the expression level
in *E. coli* is very low. Polyclonal antibodies against the C-
terminal half of hPSM have been induced in rabbits. Efforts
20 have been made in order to implement different expression tags
(His-tag and maltose binding protein fusion) as well as ex-
pression systems alternative to *E. coli* inclusion bodies.
Recombinant wild type and/or autovaccine hPSM has been de-
tected in transfected *Pichia pastoris* and mammalian cells.
25 Useful considerations regarding the DNA vaccine technology
have been made, and a preliminary feasibility study was per-
formed. DNA vaccination experiments with hPSM autovaccine
molecules are ongoing and show promising preliminary results.
Different *in vitro* assays useful for testing of and selection
30 between the mutated PSM constructs is established, including
immunochemical assays and FACS analysis. Mouse tumor cells
have been stably transfected with full length wild type hPSM

and FACS sorted for hPSM surface expression. Clones of these
cell lines have been obtained. *In vivo* xenogenic tumor models
in mice is being evaluated using these hPSM bearing syngeneic
mouse tumor cells. An array of T cell proliferation assays
5 have been performed in order to select the mouse strains for
the tumour models. CTL assays are being optimized, and con-
vincing results with model antigens have been obtained using
different immunization methods and assay conditions. Further-
more, tools necessary to study breaking of tolerance to mouse
10 PSM by immunization against mouse PSM autovaccines are being
established.

EXAMPLE 2

Production of a Her2 autovaccine

A human autovaccine against Her2 can be developed through
15 modification of the molecule by insertion of one or more
promiscuous foreign T cell epitopes to reveal a panel of
immunogenised Her2 molecules. These modified proteins will be
tested for their ability to induce antibodies which are cross-
reactive with the native parts of the Her2 molecule. Subse-
20 quently, in several *in vitro* assays and *in vivo* animal models,
the efficacy of the different constructs (as may be the case
with the DNA vaccinnation) and modified proteins will be
evaluated. The induction of specific CTL responses against
Her2 bearing tumour cells will be analysed. Also, the induced
25 antibodies will be tested for their ability to activate com-
plement via the classical pathway and to initiate ADCC via Fc-
receptors. Finally, the different modified molecules will be
tested in animal models of human breast cancer to examine
their effects on the treatment of tumours.

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Immunogenic rat and human molecules will be constructed with promiscuous T-cell epitopes at different positions in the molecule.

During vaccination against the entire extracellular domain of
5 Her2 there is a possibility of some degree of cross reaction of the antibodies with other EGFr receptors since some of these receptors are homologous by up to 40-46% in the extracellular domains. Therefore it is planned that the conserved regions of Her2 would be disrupted by inserting foreign
10 T cell epitopes at least in some of the modified proteins (see below for details).

Regions of Her2 that may potentially be CTL or B-cell epitopes are avoided in designing of constructs are seen in Fig. 3. The rationale for using these positions is as follows:

15 The human Her2 sequence can be divided into a number of domains based solely on the primary structure of the protein.

Extracellular (receptor) part:

1-173:   Domain I (N-terminal domain of mature polypeptide).

20 174-323:   Domain II (Cysteine rich domain, 24 cysteine residues).

324-483:   Domain III (ligand binding domain i the homologous EGF receptor).

484-623:   Domain IV (Cysteine rich domain, 20 cysteine resi-
25   dues).

624-654:   Transmembrane domain (TM resudues from 654 - 675).

Intracellular (kinase) part:

655-1010:   Tyrosine kinase domain (core TK domain from 725 -
             992).

1011-1235:   C-terminal domain.

5 Selection of sites in the amino acid sequence of HER2 to be
   displaced by either the P2 or P30 human T helper epitopes has
   been done considering the following parameters (loosely pri-
   oritised):

        1. Known and predicted CTL epitopes
10   2. Homology to related receptors (EGFR in particular)
        3. Conservation of cysteine residues
        4. Predicted loop, α-helix and β-sheet structures
        5. Potential N-glycosylation sites
        6. Prediction of exposed and buried amino acid residues
15   7. Domain organisation

   The CTL epitopes appear to be clustered in domain I, domain
   III, the TM domain and in two or three "hot spots" in the TK
   domain. According to the invention, these should be largely
   conserved.

20 Regions with a high degree of homology with other receptors
   are likely to be structurally important for the "overall"
   tertiary structure of Her2, and hence for antibody recog-
   nition, whereas regions with low homology possibly can be
   exchanged with only local alterations of the structure as the
25 consequence.

   Cysteine residues are often involved in intramolecular disul-
   phide bridge formation and are thus crucial for the tertiary
   structure and should preferably not be changed.

Regions predicted to form α-helix or β-sheet structures should preferably be avoided as insertion points of foreign epitopes, as these regions are probably important for the folding of the protein.

5 Potential N-glycosylation sites should preferably also be conserved because mannosylation of the protein (for example by expression in yeast) is desired, cf. the presence of mannose receptors on APCs.

Regions predicted (by their hydrophobic properties) to be
10 interior in the molecule preferably should be conserved as these could be involved in the folding. In contrast, solvent exposed regions could serve as candidate positions for insertion of the model T_H epitopes P2 and P30.

Finally, the domain organisation of the protein has also been
15 taken into consideration because of its relevance for protein structure and function.

The focus of the strategy has been to conserve the structure of the extracellular part of Her2 as much as possible, because this is the part of the protein which is relevant as target
20 for neutralising antibodies. By contrast, the intracellular part of native membrane bound Her2 on the surface of cancer cells is inaccessible for the humoral immune system.

Hence, only the presence of CTL epitopes gives reason to include this part in a vaccine. It is therefore obvious to
25 place one or more epitopes here. If it turns out that it is impossible to express the full length Her22 molecule in *E. coli* or in yeast, the intracellular part could be truncated after the first CTL epitope "hot spot" (around position 800). Additional CTL epitopes can hereafter be added to the C-termi-
30 nal end of the truncated molecule.

The transmembrane region probably is an independent folding
unit and substitution of this with T_H epitopes such as P2 or
P30 will probably not affect the HER2 structure and folding.
In addition, the TM domain might cause great problems for the

5 expression in yeast and coli and should in any case be
susbstituted. Thus, an epitope should preferably be placed in
this domain in all constructions (perhaps leaving it intact in
1 construction as it contains several CTL epitopes and because
it is somehow involved in transmission of signals upon ligand

10 binding).

The extracellular domain could principally be kept intact by
placing P2 and P30 in the intracellular and transmembrane
domains, thereby maximising the number of potential B-cell
epitopes and interfering as little as possible with the struc-

15 ture. However, the high degree of homology to EGFR and Her-3
and Her-4 make a risk for cross reactivity to these receptors
which may (or may not) be undesirable. In addition, some
monoclonal antibodies have been described which function as
agonists for the receptor (perhaps by stimulating

20 heterodimerisation or ligand binding) and increase tumour
size, *in vivo*. Positions in the extracellular domain have
therefore been selected which thereby hopefully will reduce
these risks.

This selection has involved all of the before mentioned para-

25 meters and has been based on two different assumptions: (i)
Insertion in non-conserved (with respect to related receptors)
regions will help to maintain the tertiary structure and might
reduce unwanted activation by antibodies. (ii) Insertion in
the well conserved regions can alter the structure, but might

30 at the same time reduce the risk of cross reactivity by de-
stroying the most related sequences. Both assumptions are
speculative, but as it is very difficult to predict the effect

of placing an epitope in any position of the protein some of
these positions have been included in the constructions.

It has been speculated that it could be an advantage to remove
the two cysteine rich domains completely. These are predicted
5   to form solvent exposed loop structures and could form inde-
pendent folding units perhaps involved in dimerisation (as
indicated by the many cysteines that could serve to keep a
rigid structure necessary to form a dimerisation domain).
Deleting these structures might therefore eliminate the risk
10 of activation by antibodies as well as reduce the number of
cross reacting antibodies as these domains are the most well
conserved of the extracellular part of the protein. In addi-
tion, such cysteine risch domains could be problematic to
produce in *E. coli* or yeast cells.

15 The details of constructs are as follows using the P2 and P30
epitopes as non-limiting examples: the P2 epitope will prima-
rily be placed in the extracellular domain of Her2 in combina-
tion with the P30 epitope substituting part of or all of the
membrane spanning region. The P2 epitope will be placed in
20 regions based on the criteria discussed above. The preferred
constructs have the following structures:

Construct name	Position of P2	Position of P30	Length
hHER2MA2-1A	59-73	632-652	795
hHER2MA2-2A	103-117	632-652	795
hHER2MA2-3A	149-163	632-652	795
hHER2MA2-4A	210-224	632-652	795
hHER2MA2-5A	250-264	632-652	795
hHER2MA2-6A	325-339	632-652	795
hHER2MA2-7A	369-383	632-652	795
hHER2MA2-8A	465-479	632-652	795
hHER2MA2-9A	579-593	632-652	795
hHER2MA2-1B	59-73	661-675	795
hHER2MA2-2B	103-117	661-675	795
hHER2MA2-3B	149-163	661-675	795

Construct name	Position of P2	Position of P30	Length
hHER2MA2-4B	210-224	661-675	795
hHER2MA2-5B	250-264	661-675	795
hHER2MA2-6B	325-339	661-675	795
hHER2MA2-7B	369-383	661-675	795
hHER2MA2-8B	465-479	661-675	795
hHER2MA2-9B	579-593	661-675	795
hHER2MA2-1Y	59-73	710-730	795
hHER2MA2-2Y	103-117	710-730	795
hHER2MA2-3Y	149-163	710-730	795
hHER2MA2-4Y	210-224	710-730	795
hHER2MA2-5Y	250-264	710-730	795
hHER2MA2-6Y	325-339	710-730	795
hHER2MA2-7Y	369-383	710-730	795
hHER2MA2-8Y	465-479	710-730	795
hHER2MA2-9Y	579-593	710-730	795
hHER2MA2-Z	695-709	710-730	795
hHER2MA2-C	653-667	632-652	795
hHER2MA2-BX	695-709	661-675	795
hHER2MA2-AX	695-709	632-652	795
hHER2MA2-4E	210-224	5-25	795
hHER2MA2-6E	325-339	5-25	795
hHER2MA2-8E	465-479	5-25	795
hHER2MA5-4D	210-224	632-652*	666
hHER2MA5-6D	325-339	632-652*	666
hHER2MA5-8D	465-479	632-652*	666
hHER2MA6-C	653-667	632-652	702

Position of the epitope indicates the first and the last amino
acid position of the epitope relative to the start point of
mature Her2. Length is the length in amino acids of the com-
plete construct. In all constructs except those were position
is indicated with *, the epitope substitutes an amino acid
strecth of the same length as the epitope. "*" Indicates that
the epitope is inserted rather than substituted into Her2. All
constructs listed above are therefore truncates of mature
Her2, where the omitted part is from the C-terminus.

Most of the constructions exist in different versions, e.g. in
pcDNA3.1+ vector in fusion with the natural HER2 signal pep-

tide sequence, in the vector pMT/BiP/V5-His-A as a fusion with
the BiP leader peptide for expression in Drosophila cells and
without leader sequence in the pET28b vector for expression in
E. coli cells.

5   Below are described the models that are intended for use in
    the screening and selection of modified Her2 proteins.

    1.   Induction of antibodies in transgenic rat Her2 mice and in
         rabbits to rat and human Her2, respectively, will be inves-
         tigated by conventional ELISA technology after at least
10   three immunisations. Commercially available antibodies to
         human and rat Her2 will be used as positive controls.

    2.   These rabbit antibodies will be used to study the putative
         inhibition of growth of human and transgenic mouse tumour
         cells overexpressing Her2 in an *in vitro* model.

15 3.   T cell proliferation of PBL from tetanus immunised patients
         towards selected human Her2 molecules will be investigated
         by conventional methods.

    4.   The ability of modified rat Her2 molecules to induce CTL
         responses in rat Her2 transgenic mice will be studied using
20   tumours from these mice as targets.

    5.   It is intended to synthesise a selected set of peptides in
         the transmembrane region of human Her2 encompassing P2 and
         P30 epitopes. These peptides will be tested in prolifera-
         tion of PBL from humans previously immunized with tetenus
25   toxoid to determine whether P2 and P30 epitopes could be
         efficiently processed out from within the Her2 sequences
         and presented to T cells.

6.   It is quite possible that selected human modified Her2
     proteins will be tested to generate neutralising antibodies
     in a mouse that has been genetically constructed to only
     expresses human VDJ genes. Such a mouse is available from
5   Abgenix, Fremont, CA, U.S.A. as a collaboration.

Four well-characterised transgenic mouse models for breast
cancer that contains rat Her2 oncogene have been described.
The first three transgenic mice express activated Her2 onco-
gene while the fourth model utilises inactivated Her2. All
10 models utilise an MMTV promoter to drive expression in mammary
glands.

We have decided to use two transgenic mice models: 1) a more
aggressive tumour model described by Muller et al using acti-
vated Her2 oncogene (*Muller et al, 1989*) and 2) a less ag-
15 gressive tumour model in which inactivated Her2 is used to
create focal mammary tumours with long latency (*Guy et al,
1992*). Both transgenic mice are purchased from Jackson and/or
Charles Rivers Laboratories.

In the initial experiments, these mice are allowed to produce
20 antibodies and CTL responses by immunising and boosting with
modified rat Her2 proteins. Incidence of tumours will then be
investigated as described by others (*Muller et al, 1989; Guy
et al, 1992;* Katsumata et al, 1995). Antibody levels will be
measured by an ELISA assay. The CTL activity would be deter-
25 mined by generating target cells expressing rat Her2 as men-
tioned above.

Alternatively, the nude mouse xenograft carcinoma model can be
used for passive vaccination experiments. Nude mice can be
transplanted with human tumours and inhibition of tumours
30 could be attempted with passive transfer of serum from normal
or humanised mice immunised with modified Her2 proteins. While

this would be useful for studying the role of antibody in
suppressing tumours, CTL activity cannot be directly measured
in this system.

In the second *in vivo* model, tumours in mice would also be
5 generated by transplanting cells lines from tumours of trans-
genic mice described above. Cell lines generated from these
mice would be transferred into relevant mouse strain and
localisation established using standard protocols.

Transfer of mouse tumours cells over expressing rat Her2:
10 In this system, cells will be transfected with rat genes and
transferred into MHC compatible mice. Inhibition of tumour
growth would be achieved by generating anti-Her2 responses.

In these systems; modified Her2 proteins would be used as
vaccine in adjuvants to generate antibodies and CTL responses.

15 DNA vaccination has been used successfully in several systems
to mount an effective immune response. We are currently inves-
tigating means of DNA delivery using modified self proteins.
It is our intention to utilise DNA vaccination approach to
determine effects of modified Her2 constructs in inhibiting
20 tumours in transgenic mouse models of breast cancer. Similar
approach can than possibly be applied in humans for the treat-
ment of this disease.

EXAMPLE 3

Production of an anti-FGF8b vaccine

25 In the following it will be described how a human autovaccine
against FGF8b can be developed through modification of the
molecule by insertion of one or more promiscuous foreign T

cell epitopes to reveal a panel of immunogenized "FGF8b" molecules. The constructs will be tested for their ability to induce antibodies that are cross-reactive with the authentic parts of the FGF8b molecule. Subsequently, in several *in vitro*

5   assays and *in vivo* animal models the efficacy of the different constructs will be evaluated. The induced antibodies will be tested for their ability to activate complement via the classical pathway and to initiate ADCC via Fc-receptors. Finally, the different molecules will be tested in animal models of

10 human prostate and breast cancers.

Construction of an autovaccine against FGF8b

Due to the complete identity of the murine and human FGF8b polypeptides, all constructs can be used for experiments in both humans and mice.

15 The promiscuous tetanus toxoid T helper cell epitopes P2 and P30 used with success in the human TNFα vaccine will be inserted into the FGF8b polypeptide. Due to the small size of FGF8b, constructs will be made with one epitope per molecule. Other promiscuous T helper cell epitopes such as the influenza

20 haemagglutinin epitope HA(307-319) and other T-cell epitopes discussed herein could also be considered (O'Sullivan 1991).

4 different immunogenized FGF8b constructs have been made, with the epitopes distributed along the molecule. These four constructs are made on the basis of multiple and pairwise

25 alignments of the FGF family of proteins. A pairwise alignment of FGF2 and FGF8b is used as basis for an analysis of the presumed secondary structure (i.e. beta-sheet distribution) along the FGF8b molecule. The residues that are conserved between FGF2 and FGF8b does not cluster anywhere on the

30 three-dimensional structure, which indicates that there are no particular regions of the molecule that cannot be replaced

without having deleterious effects on the folding capabili-
ties. The amino acid residues in FGF2 that align to the cys-
teine residues in FGF8b are positioned very close to each
other three-dimensionally, indicating that they form a
5 disulfide bond in FGF8b, and that the alignment is correct.
The flexibility of the N-terminal part of FGF2 was also con-
sidered.

The variant of FGF8b with the P30 epitope in the N-terminal
(F30N) was designed on the basis of no-gap alignments of the
10 amino acid residues of the FGF8b protein and the P30 epitope
(SEQ ID NO: 14), and scoring the different positions with
regard to chemical properties of every amino acid residue.
Only the region N-terminally of the predicted beta-barrel
structure was considered. In the case of F30N, there are 9
15 similar out of 21 residues. Using this pseudo-algorithm, the
substitutions would be expected to result in minimal overall
structural changes. The sequences of the four different con-
structs, as well as three-dimensional representations of the
replaced amino acids are shown in Figure 6.

20 The variant of FGF8b with the P2 epitope (SEQ ID NO: 12) in
the C-terminal (F2C) was initially designed as F30N. There is,
however, predicted a good Kd epitope at positions 195-203.
Therefore, the P2 epitope is inserted just C-terminal of this
epitope. Again, only the region C-terminal of the predicted
25 beta-barrel was considered.

The internal variants of FGF8b (F30I and F2I) were constructed
by replacing external loops in the FGF2 structure with the
epitopes P2 and P30, respectively, whereby the beta-barrel
structural backbone of the FGF structure presumably will
30 remain unchanged.

The immunogenized FGF8b molecules have been expressed in *Eschericia coli*, which facilitates large scale production of the proteins at minimal costs. Although, FGF8b contains two potential N-glycosylation sites (Asn31 and Asn177), bacteri-

5   ally expressed recombinant FGF8b has been shown to be biologi-cally active (MacArthur 1995a, Blunt 1997). In order to faci-litate purification and refolding, the FGF8b variants have been produced in a His-tagged version, thereby rendering coupling to a Ni-charged column possible.

10   Purification of the molecules has been performed utilizing the high positive charge of the protein molecules or the His-tag, and refolding will be performed using standard procedures taking the formation of the disulfide bridge into account.

The four immunogenized molecules have also together with the

15   wild type FGF8b cDNA been inserted into DNA vaccination vec-tors.

Screening and selection of the modified FGF8b molecules

The four immunogenized FGF8b molecules have been expressed in bacteria and subsequently purified from inclusion bodies. In

20   parallel, the constructs will be used as DNA vaccines. The different constructs will then be compared for their ability to induce various effects, which are desired in the treatment of prostate and breast cancer patients. Such investigations will be performed using several different *in vitro* and *in vivo*

25   assays. Finally, the results of the experiments will form the basis for the ultimate selection of one or two candidates for a FGF8b vaccine in humans.

in vitro **models**

Analyses in the murine system

Mice of different haplotypes as well as rabbits will be immu-
nized with the different FGF8b constructs in Complete Freund's
5 Adjuvant and subsequently boosted at least twice with the same
antigens emulsified in Incomplete Freunds Adjuvant. Thus, the
ability of the different constructs to break B-cell tolerance
can be compared. DNA vaccination will be performed on other
animals using purified DNA in Complete Freund's Adjuvant /
10 Incomplete Freund's Adjuvant injected intra muscularly with 14
day intervals.

Serum samples will be obtained at several time points during
the immunization schedule, and the ability of the different
constructs to induce FGF8b specific antibodies will be deter-
15 mined using a conventional ELISA method (Rochon 1994). A
commercial polyclonal antiserum, as well as a commercial
monoclonal antibody raised against FGF8b (R&D), would be used
for positive controls. The FGF8b protein is commercially
available (R&D) but will also be produced along with the other
20 FGF8b constructs and subsequently purified/refolded. This
product can then be used for coating of plates in a direct
ELISA for testing the sera from mice/rabbits immunized with
FGF8b variant proteins.

A valuable tool for investigating the effects of vaccinating
25 against FGF8b will be a FGF8b dependent cancer cell line.
Several FGF8b positive cancer cell lines, e.g. MCF-7 or SC-3,
are described in the literature. Such a FGF8b dependent murine
cancer cell line will be identified using quantitative RT-PCR,
cell proliferation experiments, and STAT-3 phosphorylation
30 assays.

The presence of FGF8b ligated to a FGF receptor on the cell
surface will be detected with FGF8b specific antibodies in
FACS or ELISA analysis. Antibodies directed against several of
the different FGF receptors are commercially available (R&D).

5 The constructs will be compared with respect to their ability
to induce antibodies capable of activating complement lysis of
FGF8b producing / bearing cells. This can be detected with one
of the mouse tumor cell lines expressing FGF8b described
earlier or, alternatively using osmotically FGF8b-loaded
10 cells. Sera from normal or transgenic mice (see below) immu-
nized with the human FGF8b constructs will be incubated with
the cell line and subsequently incubated with fresh guinea pig
complement. Antibody mediated complement lysis of the cells
can be detected by standard procedures.
15 The ability of the induced antibodies to mediate ADCC can be
studied by measuring the 51Cr-release from labeled FGF8b
expressing cells. The effector cells will be PBMC from synge-
neic mice. For establishing the assay, it may be convenient to
use a mouse cell line capable of mediating ADCC (positive for
20 Fc(-receptors) as effector cell with an antibody against human
FGF8b.

In order to show that the FGF8b candidate vaccines do not
somehow promote autoantibody induced tumor growth we will also
perform a tumor proliferation assay. Serum samples from immu-
25 nized mice will be incubated with FGF8b expressing tumor
cells. Proliferation of the tumor cells can then be detected
by their ability to incorporate 3H-thymidine, which subse-
quently is added to the cells.

Since FGF8b is known to induce proliferation of a range of
30 mammalian cells, it will also be necessary to examine the
growth promoting effects of the variant proteins. This can be

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done using cell proliferation assays as the one used by Marsh
1999.

The biological effect of FGF8b on mammalian cells should be
neutralized by the autoantibodies. This can be demonstrated by
5   using recombinant FGF8b and e.g. NIH3T3 in cell proliferation
(and morphology changes) studies. Addition of the autoantibo-
dies should abolish the transforming activity of FGF8b.

Immunization protocol

The number of animals that are to enter a FGF8b AutoVac immu-
10 nization experiment must depend on the expected penetrance of
the disease in the model, and thus, the numbers needed to
obtain statistically significant information. The immunization
protocol must be based on the experience we have from the TNFa
AutoVac project. Various immunization protocols have been used
15 for immunizing mice with the various TNFa analogs for specific
purposes, but most experiments were performed using the fol-
lowing protocol:

1. The mice should be individually marked either by earmarks
or with transponders, 10 animals in each cage. Presumably,
20 males and females must be evaluated separately, but in any
case, we will not have both sexes in the same cage. The ani-
mals should be left to rest at least 3 days after transport
and marking.

2. Antigen 1 mg/ml in PBS buffer was emulsified with an equal
25 volume Freunds complete antigen (CFA) (Difco or Sigma). The
emulsion is checked by placing a drop of the emulsion on a
water surface and it is observed whether the drop holds to-
gether or disperses. Mixing is maintained until the drop does
not disperse.

3. The standard immunization dose is 100 µg antigen in a 100 µl volume + 100 µl of adjuvant. Thus, the total immunization volume is 200 µl, administered s.c. (sub cutaneously) over the back of the animal.

5 4. Boostings are performed 2-3 weeks after the primary immunization, and subsequently at 2-3 week intervals. The boosting/immunization material is prepared and administered exactly as the immunization material, but Freunds incomplete adjuvant is used. Probably three boosts will induce the maximal titer. 10 Thus, the highest titers will be obtained 6-9 weeks after the first immunization.

5. Bleedings are orbital bleeds of 50-100 µl usually taken before the first immunization and one week after each boosting. Tail bleeds can also be used, and 10-20 µl can be suffi- 15 cient for titre determinations.

The initiation point of the immunization program will depend on the development of the disease, and the strategy we want to adopt. Initially, we suggest that it is attempted to generate the maximal immunity as soon as possible, but it is difficult 20 to start immunizations sooner than at approx. 5 weeks of age. Hereafter, high titres should be maintained by boosting at 6-8 week intervals, after the three initial boosts. There is a potential problem if the FGF8b is necessary for the normal development of the young mouse, and therefore one could argue 25 in favor of starting the immunizations later in the adult mouse.

Analyses in the human system

In the selection between the different FGF8b constructs the ability of human antigen presenting cells to present the 30 inserted immunogenic T cell epitopes to human T cells will be

investigated. This will be done by using the same *in vitro*
processing assays for P2 and P30 presentation that were used
for the TNFa vaccine. Human T cell lines, which are specific
for P2 and P30, will be established from donors vaccinated
5 against tetanus. Antigen presenting cells (PBMCs) from the
same donors will be incubated with the different constructs
and T cell lines will be added. The level of presentation of
the inserted T cell epitopes can then be compared by measuring
the stimulation of the T cell lines.

10 **in vivo animal models**

At least three different systems can be used to monitor whe-
ther the induced FGF8b antibodies are capable of controlling a
FGF8b dependent *in vivo* effect.

Mice will be transplanted with murine FGF8 expressing tumor
15 cells, and inhibition of tumor progression will be assayed
with autovaccination using the modified FGF8b proteins or
FGF8b DNA vaccines. The ideal system involves the use of cells
isolated from murine tumors. Alternatively, we will use other
murine cell lines (e.g. Balb/3T3) stably transfected with the
20 FGF8b cDNA in an expression vector.

The mouse xenograft carcinoma model will be used for passive
vaccination experiments. Nude mice will be transplanted with
human tumors, and inhibition of tumors would be attempted with
transfer of serum from normal or humanized mice immunized with
25 modified FGF8b proteins or FGF8b DNA vaccines. This would be
very useful for studying the ability of the raised antibodies
to suppress tumors.

Another approach to achieve proof of concept will involve the
use of mice transgenic for FGF8b. These mice, that are carry-
30 ing the FGF8b cDNA under control of the very specific mouse

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mammary tumor virus (MMTV) promoter, are shown to spontane-
ously develop FGF8b expressing mammary tumors (Coombes per-
sonal communication). Autovaccination of these mice with the
FGF8b variant proteins or FGF8b DNA vaccines would enable us
5 to show if the autovaccine will enable the mice to suppress or
reject the tumors.

A possible approach to obtain proof of concept would be to use
the Wnt-1 transgenic mice (MacArthur 1995c). Induction of
breast cancers by MMTV virus is reported to activate FGF8
10 expression in more than half of the mice developing tumors.
FGF8b-dependency of the tumors, could be established if our
autovaccine(s) could suppress the incidence or growth rate of
the tumors.

The fact that transgenic mice often show non-physiological
15 immunological tolerance patterns will most likely not affect
this project since the FGF8b polypeptides are identical for
human and mouse.

When, a beneficial effect of the FGF8b immunizations even-
tually has been demonstrated in the mouse model and suitable
20 human vaccine candidates have been selected it will be possi-
ble to perfom a limited number of toxicology studies. Subse-
quently, to obtain a final proof of concept, vaccine studies
on breast, and prostate cancer patients can be carried out.

Importantly, if the experiments using *in vivo* models have
25 positive outcome, more mutants will be constructed based on
the data available.

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EXAMPLE 4

Preparation of MUC-1 analogue

Only one MUC-1 autovac molecule has been made. This comprises,
in total, nine mucin repeats each having the sequence SEQ ID
5   NO: 33. The construction starts with three such sequences,
followed by a P2 epitope, followed by three more mucin se-
quences, followed by a P30 epitope, ended by three mucin
sequences.

The construction has been made with and without an N-terminal
10 UNI-his tag (SEQ ID NO: 23). Both variants have been expressed
in E. coli. The identity of the expressed protein has been
confirmed both by Western blotting and N-terminal sequencing.
The protein is expressed in soluble form, but as a dimer which
is somewhat surprising.

15 The HIS-tagged MUC-1 molecule has been purified by metal
affinity chromatography. The amount of pure protein and the
purity is currently unknown.

EXAMPLE 5

Breaking of autotolerance in a murine model system

20 CTL experiments where mice have been immunised with dendritic
cells pulsed with both a class I and a class II epitope have
previously shown an enhanced CTL induction when immunising as
well as restimulating *in vitro* with both a class I and a class
II peptide compared to an immunisation and re-stimulation with
25 just a class I epitope. This situation is comparable with
immunisation with an autovaccine, where a foreign class II
epitope is inserted in a self protein. Uptake and processing

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of these molecules by professional antigen precenting cells such as dendritic cells, leads to presentation of the foreign class II epitope together with some self class I epitopes. It is known that it is possible to elicit autoreactive CTL's, but 5 the presence of a foreign class II helper epitope very likely should enhance this CTL induction.

The potential advantage of the present invention for induction of self reactive CTLs is currently being investigated in ovalbumin transgenic mice. There exist four different trans-
10 genic lines with different ovalbumin expression levels and tolerance states, cf. Kurts C *et al.* 1997, J. Exp. Med. **186**: 239-245 disclosing the RIP-mOVA transgenic mouse (expressing ovalbumin in pancreas, kidney and thymus and having a high degree of tolerance) and Kurts C *et al.*, 1998, J. Exp. Med.
15 **188**: 409-414 disclosing the RIP-OVA^{low} and RIP-OVA^{high} transgenic mice, having low and high expression of ovalbumin, respec-tively. The last line, RIP-OVA^{int} which expresses ovalbumin at an intermediary level has been obtained from Dr. William R. Heath, co-author of the two above-mentioned refernces.

20 In the body there are different degrees of tolerance to dif-ferent antigens. One of the least degrees of tolerance is found on circulating antigens in large amounts. These antigens will all enter thymus, where self reactive T-cells are de-leted. These antigens are under "central tolerance". Tissue
25 specific antigens, on the other hand, do not directly enter the thymus and is generally under "peripheral tolerance", exerted by e.g. T-cell anergy.

Two ovalbumin AutoVac constructs have produced. They both relate to the sequence with accession No: J00845 in EMBL where
30 the sequence from P30 (SEQ ID NO: 14) have been inserted in two different positions.

In construct "OVA 3.1", P30 is inserted in the position that correspond to amino acid nos. 272-292 in ovalbumin. In construct "OVA 3.2", P30 is inserted in the position that corresponds to amino acid nos. 321-341 in ovalbumin. These con-
5 structs have been inserted in the vector pVax1 and used for DNA immunisation.

Mice have been immunised intradermally once with 100 ug each of DNA. Three weeks after this immunisation, the spleens were removed and a CTL assay was set up using target cells expres-
10 sing the dominant ovalbumin epitope SIINFEKL and the scrambled FILKSINE peptide as control. The immunizations provided a clear CTL induction in wild-type C57BL/6 mice - as expected, since both ovalbumin and P30 are foreign in these mice.

We now intend to immunise the 4 lines of ovalbumin transgenic
15 mice with these AutoVac constructs. The RIP-OVA^{low}, RIP-OVA^{int}, and RIP-OVA^{high} express increasing amounts of ovalbumin and have different degrees of tolerance and, as mentioned above, also RIP-mOVA has a high degree of tolerance.

In these 4 lines of transgenic mice, only P30 will be foreign.
20 Ovalbumin is a self-antigen in these mice and this situation will therefore constitute a true autovaccination for CTL induction towards ovalbumin.

Preliminary results obtained in RIP-OVA^{low} mice having the lowest degree of "peripheral tolerance" to ovalbumin demon-
25 strated that both the ovalbumin with inserted P30 and the naturally occurring ovalbumin molecules were capable of inducing CTL responses - it is expected that transgenic mice having higher degrees of tolerance will only be capable of mounting a CTL response against the modified ovalbumin mole-
30 cules and not the naturally occurring form.

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CLAIMS

1. A method for inducing an immune response against a polypep-
tide antigen in an animal, including a human being, said
polypeptide antigen being weakly immunogenic or non-immunogen-
5 ic in the animal, the method comprising effecting simultaneous
presentation by antigen presenting cells (APCs) of the ani-
mal's immune system of an immunogenically effective amount of
   1)   at least one CTL epitope derived from the polypeptide
        antigen and/or at least one B-cell epitope derived from the
10   cell-associated polypeptide antigen, and
   2)   at least one first T helper cell epitope (T_H epitope) which
        is foreign to the animal.

2. A method for down-regulating a cell-associated polypeptide
antigen in an animal, including a human being, said polypep-
15 tide antigen being weakly immunogenic or non-immunogenic in
the animal, by inducing a specific cytotoxic T-lymphocyte
(CTL) response against cells carrying the cell-associated
polypeptide antigen on their surface or harbouring the cell-
associated polypeptide antigen in their intracellular compart-
20 ment, the method comprising effecting, in the animal, simulta-
neous presentation by a suitable antigen presenting cell (APC)
of
   1)   at least one CTL epitope derived from the cell-associated
        polypeptide antigen, and
25 2)   at least one first T-helper lymphocyte (T_H) epitope which
        is foreign to the animal.

3. The method according to claim 1 or 2, wherein said at least
one CTL epitope when presented is associated with an MHC Class
I molecule on the surface of the APC and/or wherein said at
30 least one first foreign T_H epitope when presented is associated
with an MHC Class II molecule on the surface of the APC.

4. The method according to any one of the preceding claims, wherein the APC is a dendritic cell or a macrophage.

5. The method according to any one of the preceding claims, wherein the polypeptide antigen is selected from a tumour-associated polypeptide antigen, a self-protein, a viral polypeptide antigen, and a polypeptide antigen derived from an intracellular parasite or bacterium.

6. The method according to any one of the preceding claims, wherein presentation by the APC of the CTL epitope and the first foreign T_H epitope is effected by presenting the animal's immune system with at least one first analogue of the polypeptide antigen, said first analogue comprising a variation of the amino acid sequence of the polypeptide antigen, said variation containing at least the CTL epitope and the first foreign T_H epitope.

7. The method according to claim 6, wherein the at least first analogue contains a substantial fraction of known and predicted CTL epitopes of the cell-associated polypeptide antigen.

8. The method according to claim 7, wherein the substantial fraction of known and predicted CTL epitopes in the amino acid sequence of the analogue are recognized by at least 90% of the MHC-I haplotypes recognizing all known and predicted CTL epitopes in the cell-associated polypeptide antigen.

9. The method according to any one of claims 6-8, wherein substantially all known CTL epitopes of the cell-associated polypeptide antigen are present in the analogue and/or wherein substantially all predicted CTL epitopes of the cell-associated polypeptide antigen are present in the at least first analogue.

10. The method according to any one of claims 6-9, wherein the at least one first analogue further comprises a part consisting of a modification of the structure of the cell-associated polypeptide antigen, said modification having as a result that

5 immunization of the animal with the first analogue induces production of antibodies in the animal against the cell-associated polypeptide antigen.

11. The method according to any one of the preceding claims, which comprises effecting presentation to the animal's immune

10 system of an immunogenically effective amount of at least one second analogue of the polypeptide antigen, said second analogue containing a modification of the structure of the polypeptide antigen, said modification having as a result that immunization of the animal with the second analogue induces

15 production of antibodies against the cell-associated polypeptide antigen.

12. The method according to claim 11, wherein the modification comprises that at least one second foreign T_H epitope is included in the second analogue.

20 13. The method according to any one of claims 6-12, wherein the first and/or second analogue(s) comprise(s) a substantial fraction of the cell-associated polypeptide antigen's B-cell epitopes.

14. The method according to any one of claims 6-13, wherein

25 the variation and/or modification involves amino acid substitution and/or deletion and/or insertion and/or addition.

15. The method according to any one of claims 6-14, wherein the variation and/or modification comprises that

- at least one first moiety is included in the first and/or second analogue(s), said first moiety effecting targeting of the analogue to an antigen presenting cell (APC), and/or
- at least one second moiety is included in the first and/or

5   second analogue(s), said second moiety stimulating the immune system, and/or
- at least one third moiety is included in the first and/or second analogue(s), said third moiety optimizing presentation of the analogue to the immune system.

10 16. The method according to any one of claims 6-15, wherein the variation and/or modification includes duplication of at least one B-cell epitope or of at least one CTL epitope of the cell-associated polypeptide antigen

17. The method according to any one of claims 6-16, wherein
15 the variation and/or modification includes introduction of a hapten.

18. The method according to any one of the preceding claims, wherein the first and/or second foreign T_H epitope(s) is/are immunodominant.

20 19. The method according to any one of the preceding claims, wherein the first and/or second foreign T_H epitope(s) is/are promiscuous.

20. The method according to any one of claims 12-19, wherein the first and/or second foreign T_H epitope(s) is/are selected
25 from a natural T_H epitope and an artificial MHC-II binding peptide sequence.

21. The method according to claim 20, wherein the natural T_H epitope is selected from a Tetanus toxoid epitope such as P2

or P30, a diphtheria toxoid epitope, an influenza virus
hemagluttinin epitope, and a *P. falciparum* CS epitope.

22. The method according to any one of claims 12-21, wherein
the first and/or second T_H epitopes and/or first and/or second
5   and/or third moieties are present in the form of
    -   side groups attached covalently or non-covalently to suit-
        able chemical groups in the amino acid sequence of the
        cell-associated polypeptide antigen or a subsequence there-
        of, and/or
10 -   fusion partners to the amino acid sequence derived from the
        cell-associated polypeptide antigen.

23. The method according to claim 22, wherein the first moiety
is a substantially specific binding partner for an APC spe-
cific surface antigen such as a carbohydrate for which there
15 is a receptor on the APC, e.g. mannan or mannose.

24. The method according to any one of claims 15-23, wherein
the second moiety is a cytokine selected from interferon γ
(IFN-γ), Flt3L, interleukin 1 (IL-1), interleukin 2 (IL-2),
interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 12
20 (IL-12), interleukin 13 (IL-13), interleukin 15 (IL-15), and
granulocyte-macrophage colony stimulating factor (GM-CSF), or
an effective part thereof; a heat-shock protein selected from
HSP70, HSP90, HSC70, GRP94, and calreticulin (CRT), or an
effective part thereof; or a hormone.

25 25. The method according to any one of claims 15-24, wherein
the third moiety is a lipid such as a palmitoyl group, a
myristyl group, a farnesyl group, a geranyl-geranyl group, a
GPI-anchor, and an N-acyl diglyceride group.

26. The method according to claim any one of claims 6-25,
30 wherein the first and/or second analogue(s) has/have substan-

tially the overall tertiary structure of the cell-associated
polypeptide antigen.

27. The method according to any one of claims 6-26, wherein
presentation by the APC is effected by administering, to the
5   animal, an immunogenically effective amount of the at least
one first analogue.

28. The method according to claim 27, wherein is also admini-
stered an immunologically effective amount of the at least one
second analogue.

10   29. The method according to claim 27 or 28, wherein said at
least one first and/or second analogue(s) is/are formulated
together with a pharmaceutically and immunologically accept-
able carrier and/or vehicle and, optionally an adjuvant.

30. The method according to claim 29, wherein said adjuvant
15   facilitates uptake by APCs, such as dendritic cells, of the at
least first and/or second analogues.

31. The method according to claim 30, wherein the adjuvant is
selected from the group consisting of an immune targeting
adjuvant; an immune modulating adjuvant such as a toxin, a
20   cytokine, and a mycobacterial derivative; an oil formulation;
a polymer; a micelle forming adjuvant; a saponin; an
immunostimulating complex matrix (ISCOM matrix); a particle;
DDA; aluminium adjuvants; DNA adjuvants; γ-inulin; and an
encapsulating adjuvant.

25   32. The method according to claim 31, wherein the cytokine is
as defined as in claim 24, or an effective part thereof,
wherein the toxin is selected from the group consisting of
listeriolycin (LLO), Lipid A (MPL, L180.5/RalLPS), and heat-
labile enterotoxin, wherein the mycobacterial derivative is

selected from the group consisting of muramyl dipeptide, complete Freund's adjuvant, RIBI, and a diester of trehalose such as TDM and TDE, wherein the immune targeting adjuvant is selected from the group consisting of CD40 ligand, CD40 anti-

5 bodies or specifically binding fragments thereof, mannose, a Fab fragment, and CTLA-4, wherein the oil formulation com- prises squalene or incomplete Freund's adjuvant, wherein the polymer is selected from the group consisting of a carbohy- drate such as dextran, PEG, starch, mannan, and mannose; a

10 plastic polymer such as; and latex such as latex beads, where- in the saponin is *Quillaja saponaria* saponin, Quil A, and QS21, and wherein the particle comprises latex or dextran.

33. The method according to any one of claims 27-32, which includes administration via a route selected from the oral

15 route and the parenteral route such as the intradermal, the subdermal, the intracutaneous, the subcutaneous; the peritone- al, the buccal, the sublinqual, the epidural, the spinal, the anal, and the intracranial routes.

34. The method according to any of claim 27-33, which includes

20 at least one administration a year, such as at least 2, 3, 4, 5, 6, and 12 administrations a year.

35. The method according to any one of claims 1-5, wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying a

25 nucleic acid fragment encoding and expressing the at least one CTL epitope and the at least one T_H epitope.

36. The method according to any one of claims 6-14, wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying at

30 least one nucleic acid fragment which encodes and expresses the at least first analogue.

37. The method according to any one of claims claims 15-26, wherein the T_H epitope and/or the first and/or second and/or third moieties are present in the form of fusion partners to the amino acid sequence derived from the cell-associated

5 polypeptide antigen, and wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying at least one nucleic acid fragment encoding and expressing the first and/or second analogue.

38. The method according to any one of claims 11-14 or 36,

10 wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying at least one nucleic acid fragment which encodes and expresses the at least second analogue.

39. The method according to claim 38, wherein the non-patho-

15 genic microorganism or virus is administered once to the animal.

40. The method according to any one of claims 1-5, wherein presentation is effected by *in vivo* introducing, into the APC, at least one nucleic acid fragment which encodes and expresses

20 the at least one CTL epitope and/or the at least one B-cell epitope, and the at least one first foreign T_H epitope.

41. The method according to any one of claims 6-14, wherein presentation is effected by *in vivo* introducing, into the APC, at least one nucleic acid fragment encoding and expressing the

25 first analogue.

42. The method according to any one of claims claims 15-26, wherein the T_H epitope and/or the first and/or second and/or third moieties are present in the form of fusion partners to the amino acid sequence derived from the cell-associated

polypeptide antigen, and wherein presentation is effected by
in vivo introducing, into the APC, at least one nucleic acid
fragment encoding and expressing the first and/or second
analogue.

5   43. The method according to any one of claims 11-14 and 41,
which further comprises *in vivo* introduction, into the APC, of
at least one nucleic acid fragment encoding and expressing the
second analogue.

10 44. The method according to any one of claims 1-5, wherein
presentation is effected by *in vivo* co-introducing, into the
APC, at least two nucleic acid fragments, wherein one encodes
and expresses the at least one CTL epitope and wherein another
encodes and expresses the at least one first foreign T_H epi-
15 tope, and wherein the first foreign T_H epitope is as defined in
any one of claims 1, 2 and 21-24.

45. The method according to any one of claims 40-44, wherein
the nucleic acid fragment(s) introduced is/are selected from
naked DNA, DNA formulated with charged or uncharged lipids,
20 DNA formulated in liposomes, DNA included in a viral vector,
DNA formulated with a transfection-facilitating protein or
polypeptide, DNA formulated with a targeting protein or
polypeptide, DNA formulated with a targeting carbohydrate, DNA
formulated with Calcium precipitating agents, DNA coupled to
25 an inert carrier molecule, and DNA formulated with an adju-
vant.

46. The method according to claim 45, wherein the adjuvant is
selected from the group consisting of the adjuvants defined in
any one of claims 30-32.

30 47. The method according to any one of claims 40-46, wherein
the mode of administration is as defined in claim 33 or 34.

48. A method for selection of an immunogenic analogue of a cell-associated polypeptide antigen which is weakly immunogenic or non-immunogenic in an animal, said immunogenic analogue being capable of inducing a CTL response in the animal

5   against cells displaying an MHC Class I molecule bound to an epitope derived from the cell-associated polypeptide antigen, the method comprising

a) identifying at least one subsequence of the amino acid sequence of the cell-associated polypeptide antigen which

10   does not contain known or predicted CTL epitopes,

b) preparing at least one putatively immunogenic analogue of the cell-associated polypeptide antigen by introducing, in the amino acid sequence of the cell-associated polypeptide antigen, at least one T_H epitope foreign to the animal in a

15   position within the at least one subsequence identified in step a), and

c) selecting the/those analogues prepared in step b) which are verifiably capable of inducing a CTL response in the animal.

20 49. The method according to claim 48, wherein

1) the subsequence identified in step a) further does not contain cysteine residues, or, alternatively, wherein the T_H epitope introduced in step b) does not substantially alter the pattern of cystein residues, and/or

25 2) the subsequence identified in step a) further does not contain known or predicted glycosylation sites, or, alternatively, wherein the T_H epitope introduced in step b) does not substantially alter the glycosylation pattern, and/or

3) the subsequence identified in step a) contributes signifi-

30   cantly to a patophysiological effect exerted by the cell-associated polypeptide antigen, and wherein the introduc-

tion in step b) of the foreign T_H epitope reduces or abolishes said patophysiological effect, and/or

4)   the subsequence identified in step a) is homologous to an amino acid sequence of a different protein antigen of the animal, and wherein the introduction of the T_H epitope in step b) substantially removes the homology, and/or

5)   introduction in step b) of the foreign T_H epitope results in preservation of a substantial fraction of B-cell epitopes of the cell-associated polypeptide antigen.

50. The method according to claim 49, variant 5, wherein the analogue has the overall tertiary structure of the cell-associated polypeptide antigen.

51. A method for the preparation of cell producing an analogue of a cell-associated polypeptide antigen, the method comprising introducing, into a vector, a nucleic acid sequence encoding an analogue which has been selected according to the method of any one of claims 48-50 and transforming a suitable host cell with the vector.

52. A method for the preparation of an analogue of a cell-associated polypeptide antigen, the method comprising culturing the cell obtained according to the method of claim 51 under conditions facilitating expression of the nucleic acid sequence encoding the cell-associated polypeptide antigen, and recovering the analogue from the culture supernatant or from the cells.

53. The method according to claim 52 which further comprises the step of purifying the recovered analogue and, optionally subjecting the purified product to artificial post-transla-

tional modifications such as refolding, treatment with en-
zymes, chemical modification, and conjugation.

54. The method according to any one of the preceding claims,
    wherein the weak cell-associated antigen is selected from the
5   group consisting of 5 alpha reductase, α-fetoprotein, AM-1 ,
    APC, APRIL, BAGE, β-catenin, Bcl2, bcr-abl (b3a2), CA-125,
    CASP-8 / FLICE, Cathepsins, CD19, CD20, CD21, CD23, CD22,
    CD33, CD35, CD44, CD45, CD46, CD5, CD52, CD55 (791Tgp72),
    CD59, CDC27, CDK4, CEA, c-myc, Cox-2, DCC, DcR3, E6 / E7,
10 EGFR, EMBP, Ena78, farsyl transferase, FGF8a or FGF8b, FLK-
    1/KDR, Folic Acid Receptor, G250, GAGE-Family, gastrin 17,
    Gastrin-releasing hormone (Bombesin), GD2 / GD3 / GM2, GnRH,
    GnTV, GP1, gp100 / Pmel 17, gp-100-in4, gp15, gp75 / TRP-1,
    hCG, Heparanase, Her2 / neu, HMTV, Hsp70, hTERT (telomerase),
15 IGFR1, IL-13R, iNOS, Ki 67, KIAA0205, K-ras, H-ras, N-ras, KSA
    (CO17-1A), LDLR-FUT, MAGE Family (MAGE-1, MAGE-2, MAGE-3,
    etc), Mammaglobin, MAP17, Melan-A / MART-1, mesothelin, MIC
    A/B, MT-MMP's, Mox1, Mucin such as MUC-1, MUC-2, MUC-3, and
    MUC-4 being abberantly glycosylated, MUM-1, NY-ESO-1,
20 Osteonectin, p15, P170 / MDR1, p53, p97 / melanotransferrin,
    PAI-1, PDGF, Plasminogen (uPA), PRAME, Probasin, Progenipoi-
    etin, PSA, PSM, RAGE-1, Rb, RCAS1, SART-1, SSX gene family,
    STAT3, STn (mucin assoc.), TAG-72, TGF-α, TGF-β, Thymosin β
    15, TNF-α, TPA, TPI, TRP-2, Tyrosinase, VEGF, ZAG, p16INK4,
25 and Glutathione S-transferase.

55. The method according to claim 54, wherein the cell-associ-
    ated polypeptide antigen is human PSM.

56. The method according to claim 55, wherein the foreign T-
    cell epitope is introduced in a part of the PSM amino acid
30 sequence defined by SEQ ID NO: 2 positions 16-52 and/or 87-108
    and/or 210-230 and/or 269-289 and/or 298-324 and/or 442-465
    and/or 488-514 and/or 598-630 and/or 643-662 and/or 672-699.

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57. The method according to claim 55 or 56 used in the treatment or amelioration of prostate cancer.

58. The method according to claim 54, wherein the cell-associ-
5 ated polypeptide antigen is fibroblast growth factor 8b
(FGF8b).

59. The method according to claim 58, where the foreign T-cell epitope is introduced in a part of the FGF8b amino acid sequence defined by SEQ ID NO: 6 positions 1-54 and/or 178-215
10 and/or 55-58 and/or 63-68 and/or 72-76 and/or 85-91 and/or 95-102 and/or 106-111 and/or 115-120 and/or 128-134 and/or 138-144 and/or 149-154 and/or 158-162 and/or 173-177, and wherein the introduction preferably does not substantially involve amino acids 26-45 and amino acids 186-215.

15 60. The method according to claim 58 or 59 used in the treatment or amelioration of cancer such as prostate cancer and breast cancer.

61. The method according to claim 54, wherein the cell-associated polypeptide antigen is Her2.

20 62. The method according to claim 61, wherein the foreign T-cell epitope is introduced in a part of the Her2 amino acid sequence defined by SEQ ID NO: 3 positions 5-25 and/or 59-73 and/or 103-117 and/or 149-163 and/or 210-224 and/or 250-264 and/or 325-339 and/or 369-383 and/or 465-479 and/or 579-593
25 and/or 632-652 and/or 653-667 and/or 661-675 and/or 695-709 and/or 710-730.

72-86 and/or 146-160 and/or 221-235 and/or 257-271 and/or 387-401.

63. The method according to claim 61 or 62 used in the treatment or amelioration of breast cancer.

64. An analogue of human PSM which is immunogenic in humans,
5 said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of PSM and including at least one foreign T_H epitope as defined in any one of claims 18-21.

65. The analogue according to claim 64, wherein the at least
10 one foreign T_H epitope is present as an insertion in the PSM amino acid sequence or as a substitution of part of the PSM amino acid sequence or as the result of deletion of part of the PSM amino acid sequence.

66. The analogue according to claim 65, wherein the foreign T_H
15 epitope is introduced in the positions defined in claim 56.

67. An analogue of human Her2 which is immunogenic in humans, said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of Her2 and including at least one foreign T_H epitope as defined in any one of claims
20 18-21.

68. The analogue according to claim 67, wherein the at least one foreign T_H epitope is present as an insertion in the Her2 amino acid sequence or as a substitution of part of the Her2 amino acid sequence or as the result of deletion of part of
25 the Her2 amino acid sequence.

69. The analogue according to claim 68, wherein the foreign T_H epitope is introduced in the positions defined in claim 62.

70. An analogue of human/murine FGF8b which is immunogenic in humans, said analogue comprising a substantial part of all

known and predicted CTL and B-cell epitopes of FGF8b and
including at least one foreign T$_H$ epitope as defined in any one
of claims 18-21.

71. The analogue according to claim 70, wherein the at least
5 one foreign T$_H$ epitope is present as an insertion in the FGF8b
amino acid sequence or as a substitution of part of the FGF8b
amino acid sequence or as the result of deletion of part of
the FGF8b amino acid sequence.

72. The analogue according to claim 71, wherein the foreign T$_H$
10 epitope is introduced in the positions defined in claim 59.

73. An immunogenic composition which comprises, as an effec-
tive immunogenic agent the analogue according to any one of
claims 64-72 in admixture with a pharmaceutically and immuno-
logically acceptable carrier or vehicle, and optionally an
15 adjuvant.

74. A nucleic acid fragment which encodes an analogue accor-
ding to any one of claims 64-72.

75. A vector carrying the nucleic acid fragment according to
claim 74.

20 76. The vector according to claim 75 which is capable of
autonomous replication.

77. The vector according to claim 75 or 76 which is selected
from the group consisting of a plasmid, a phage, a cosmid, a
mini-chromosome, and a virus.

25 78. The vector according to any one of claims 75-77, compri-
sing, in the 5'-3' direction and in operable linkage, a pro-
moter for driving expression of the nucleic acid fragment

according to claim 74, optionally a nucleic acid sequence
encoding a leader peptide enabling secretion of or integration
into the membrane of the polypeptide fragment, the nucleic
acid fragment according to claim 74, and optionally a nucleic
5 acid sequence encoding a terminator.

79. The vector according to any one of claims 75-78 which,
when introduced into a host cell, is integrated in the host
cell genome or is not capable of being integrated in the host
cell genome.

10 80. A transformed cell carrying the vector of any one of
claims 75-79.

81. A composition for inducing production of antibodies a-
gainst PSM, Her2 or FGF8b, the composition comprising
1)   a nucleic acid fragment according to claim 74 or a vector
15   according to any one of claims 75-79, and
2)   a pharmaceutically and immunologically acceptable diluent
     and/or vehicle and/or adjuvant.

82. A stable cell line which carries the vector according to
any one of claims 75-79 and which expresses the nucleic acid
20 fragment according to claim 74, and which optionally secretes
or carries the analogue according to any one of claims 64-72
on its surface.

83. A method for the preparation of the cell according to
claim 80, the method comprising transforming a host cell with
25 the nucleic acid fragment according to claim 74 or with the
vector according to any one of claims 75-79.
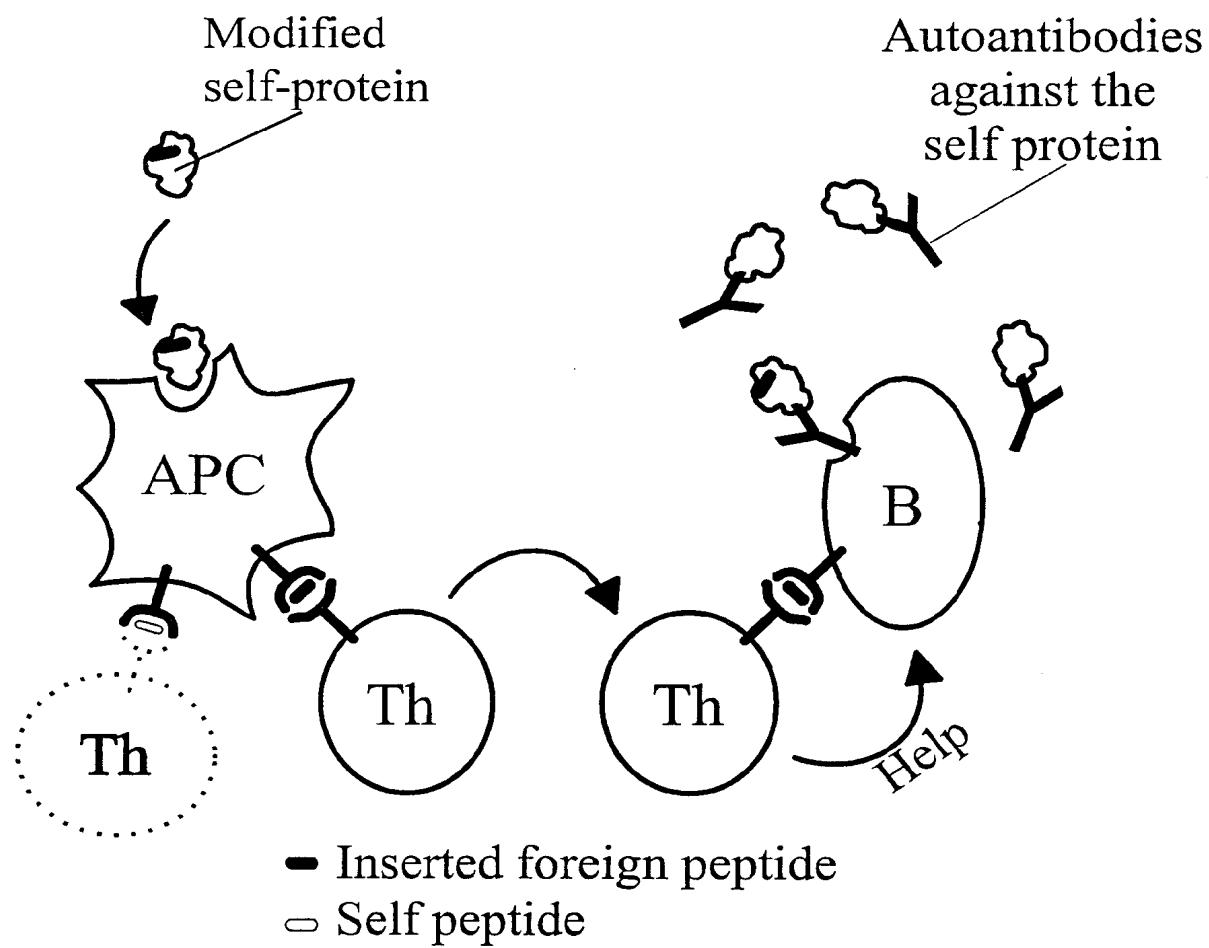
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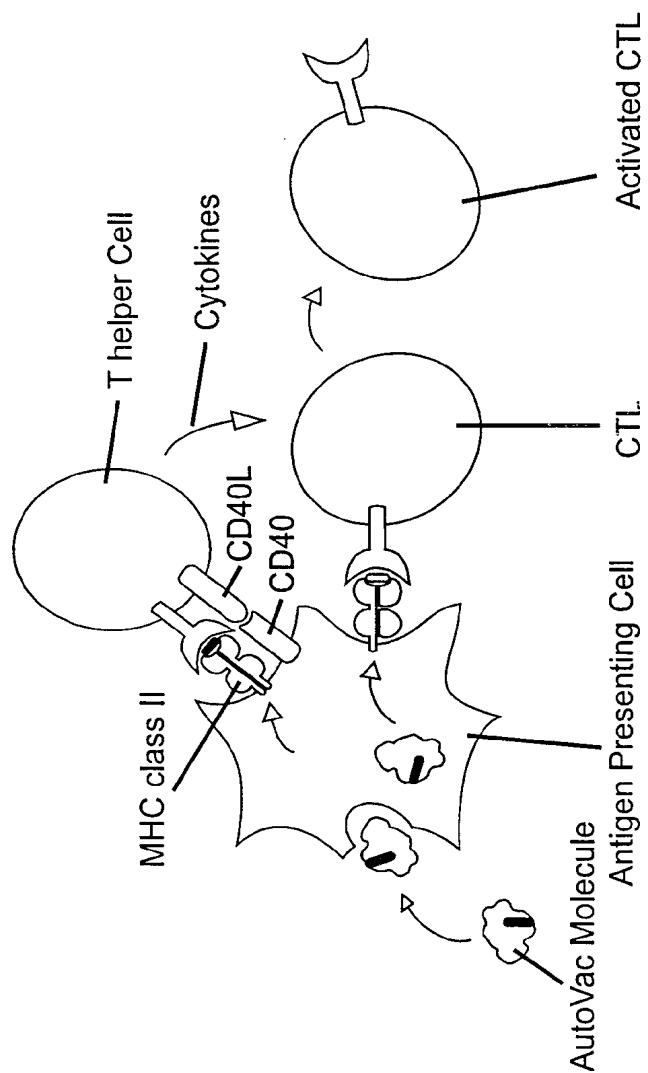
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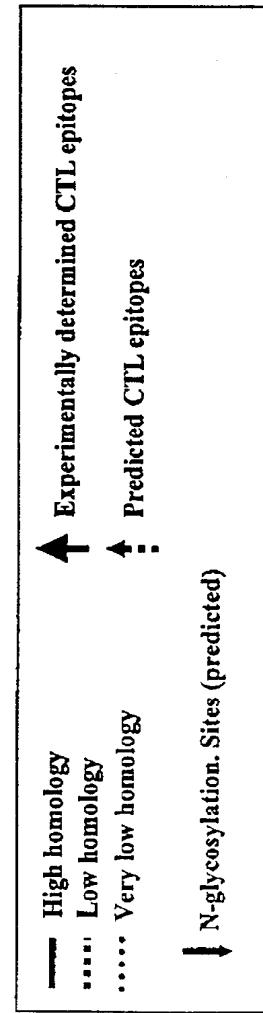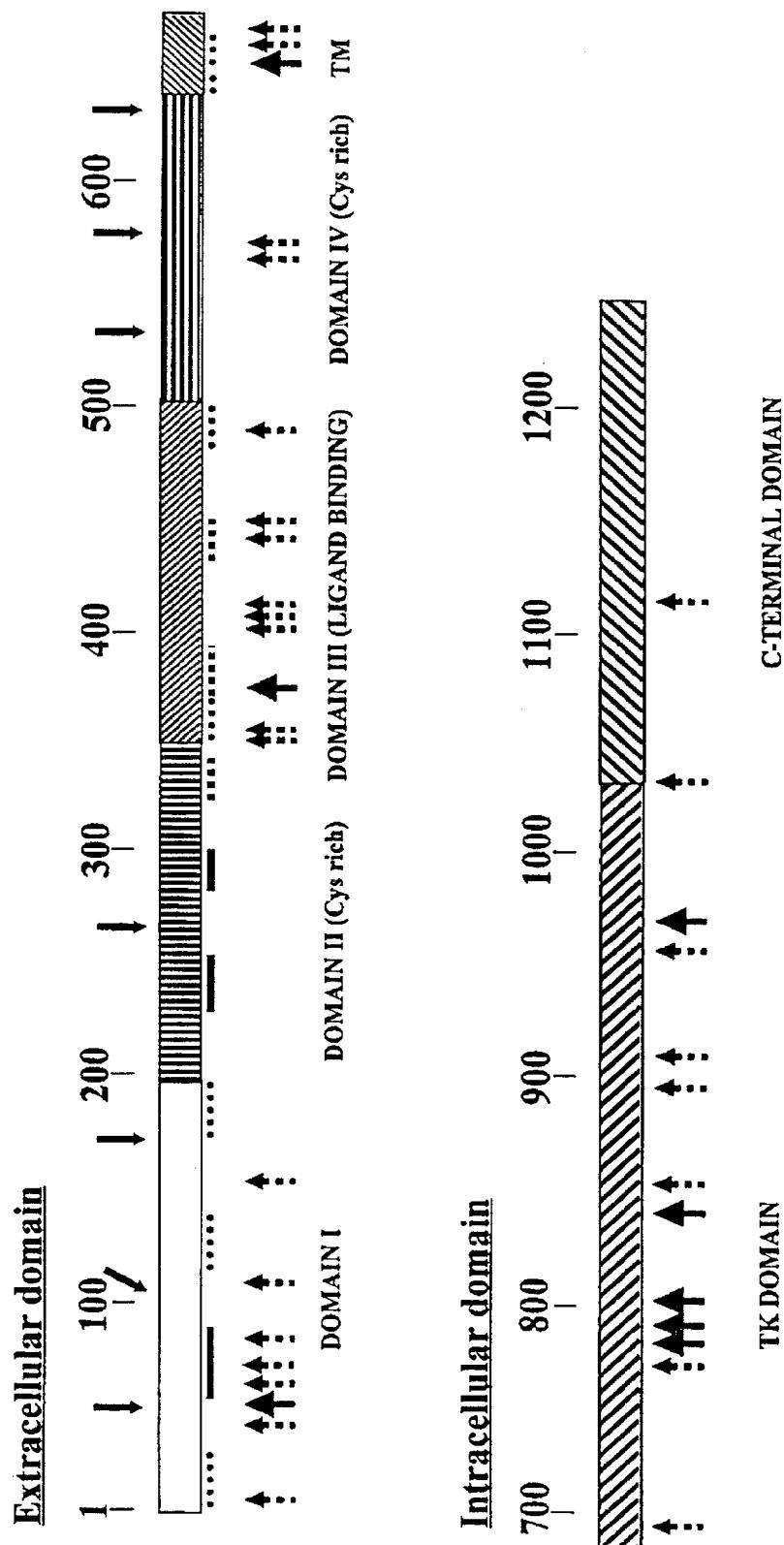
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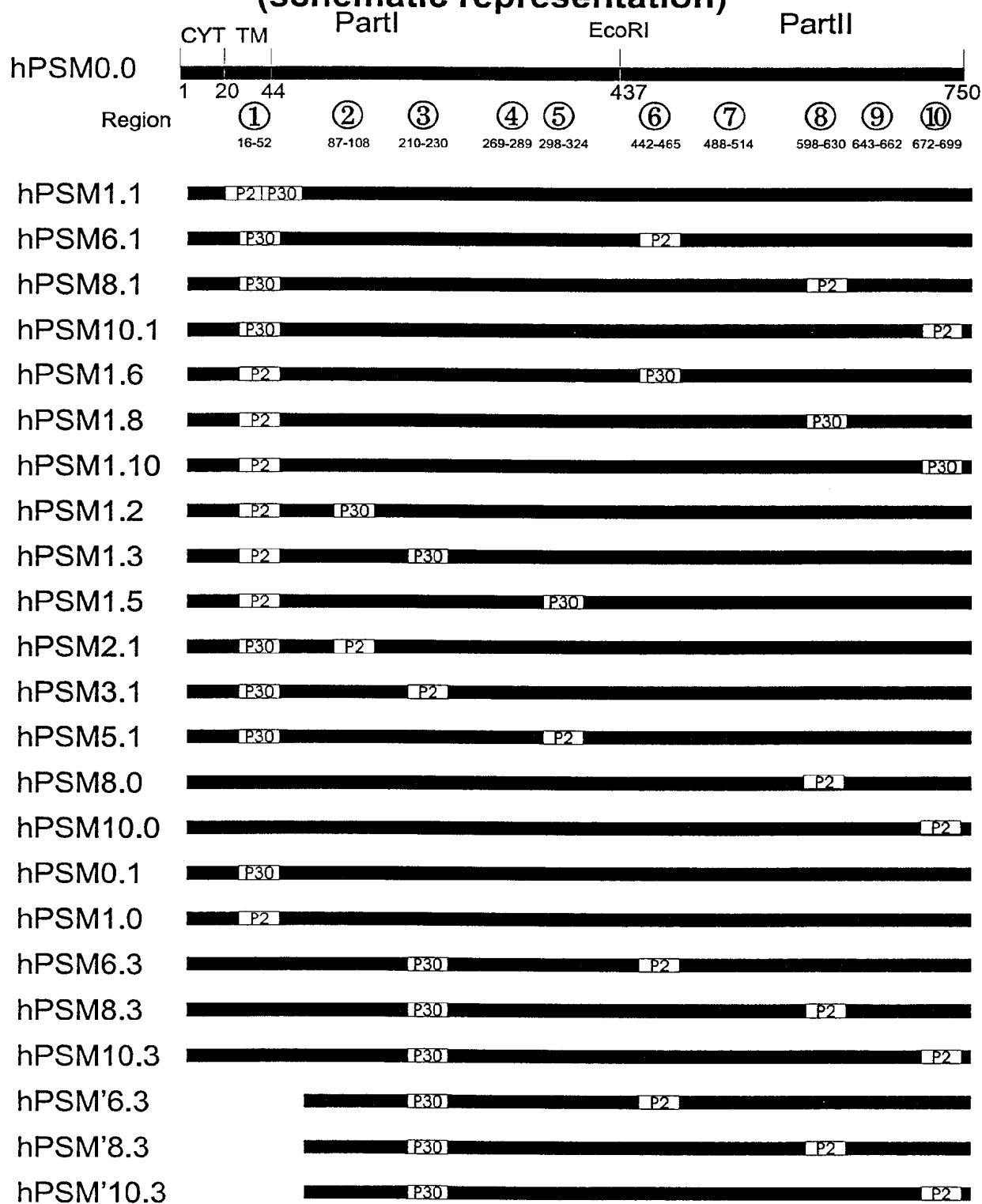
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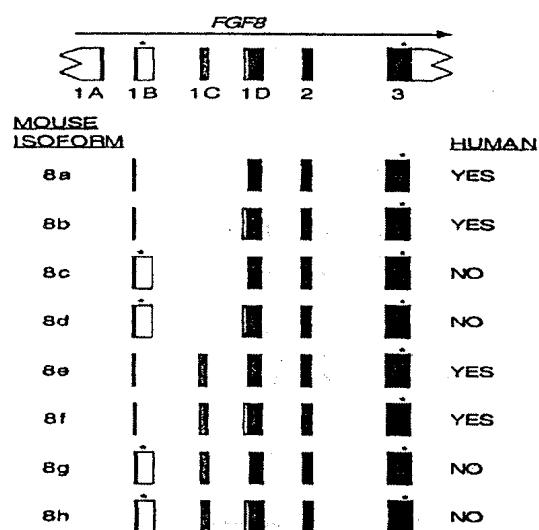
Fig. 5A

```
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FTQHVREQSL VTDQLSRRLI RTYQLYSRTS GKHVQVLANK RINAMAEDGD PFAKLIVETD  91
TFGSRVRVRG AETGLYICMN KKGKLIAKSN GKGKDCVFTE IVLENÑYTAL QNAKYEGWYM 151
AFTRKGRPRK GSKTRQHQRE VHFMKRLPRG HHTTEQSLRF EFLNYPPFTR SLRGSQRTWA 211
PEPR                                                             215
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Fig. 5B

F30N.

F2I.

F30I.

F2C.
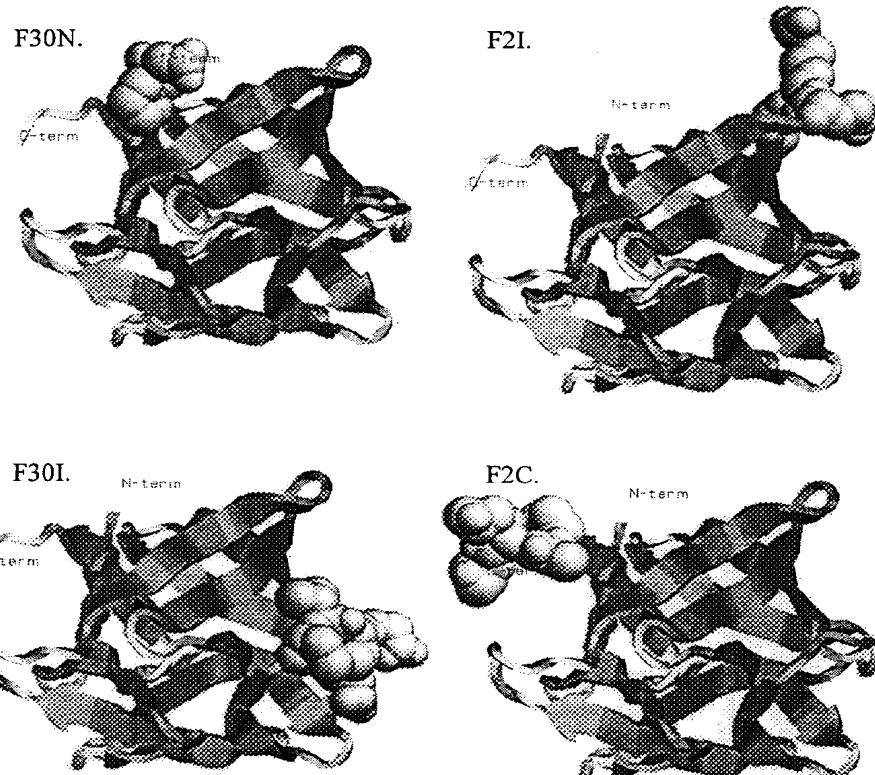
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F2I             MAQVTVQSSPNFTQHVREQSLVTDQLSRRLIRTYQLYSRTSGKHVQ  46
F30I            MAQVTVQSSPNFTQHVREQSLVTDQLSRRLIRTYQLYSRTSGKHVQ  46
F2C             MAQVTVQSSPNFTQHVREQSLVTDQLSRRLIRTYQLYSRTSGKHVQ  46

WT      VLANKRINAMAEDGDPFAKLIVETDTF              GSRVRVRGAETGLYICMNKKGKLIAK 119
F30N    VLANKRINAMAEDGDPFAKLIVETDTF              GSRVRVRGAETGLYICMNKKGKLIAK  99
F2I     VLANKRINAMAEDGDPFAKLIVETDQYIKANSKFIGITELGSRVRVRGAETGLYICMNKKGKLIAK 112
F30I    VLANKRINAMAEDGDPFAKLIVETDTF              GSRVRVRGAETGLYICMNKKGKLIAK  99
F2C     VLANKRINAMAEDGDPFAKLIVETDTF              GSRVRVRGAETGLYICMNKKGKLIAK  99

WT      SNG              KGKDCVFTEIGLENNYTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 167
F30N    SNG              KGKDCVFTEIGLENNYTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 147
F2I     SNG              KGKDCVFTEIGLENNYTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 160
F30I    SNGFNNFTVSFWLRVPKVSASHLEDCVFTEIGLENNYTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 165
F2C     SNG              KGKDCVFTEIGLENNYTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 147

WT      HQREVHFMKRLPRGHHTTEQSLRFEFLNYPPFT   RSLRGSQRTWA   PEPR 215
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F2I     HQREVHFMKRLPRGHHTTEQSLRFEFLNYPPFT   RSLRGSQRTWA   PEPR 208
F30I    HQREVHFMKRLPRGHHTTEQSLRFEFLNYPPFT   RSLRGSQRTWA   PEPR 213
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Fig. 6

1

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<223> Human PSM'

<220>
<221> misc_feature
<222> (4)..(6)
<223> ggt or tgg encoding Gly and Trp, respectively

<400> 1
atg ggt aat ctc ctt cac gaa acc gac tcg gct gtg gcc acc gcg cgc   48
Met Gly Asn Leu Leu His Glu Thr Asp Ser Ala Val Ala Thr Ala Arg
  1   5   10   15

cgc ccg cgc tgg ctg tgc gct ggg gcg ctg gtg ctg gcg ggt ggc ttc   96
Arg Pro Arg Trp Leu Cys Ala Gly Ala Leu Val Leu Ala Gly Gly Phe
              20   25   30

2

```
ttt ctc ctc ggc ttc ctc ttc ggg tgg ttt ata aaa tcc tcc aat gaa    144
Phe Leu Leu Gly Phe Leu Phe Gly Trp Phe Ile Lys Ser Ser Asn Glu
        35              40                  45

gct act aac att act cca aag cat aat atg aaa gca ttt ttg gat gaa    192
Ala Thr Asn Ile Thr Pro Lys His Asn Met Lys Ala Phe Leu Asp Glu
        50              55                  60

ttg aaa gct gag aac atc aag aag ttc tta tat aat ttt aca cag ata    240
Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr Gln Ile
65                  70              75                  80

cca cat tta gca gga aca gaa caa aac ttt cag ctt gca aag caa att    288
Pro His Leu Ala Gly Thr Glu Gln Asn Phe Gln Leu Ala Lys Gln Ile
                85              90                  95

caa tcc cag tgg aaa gaa ttt ggc ctg gat tct gtt gag cta gca cat    336
Gln Ser Gln Trp Lys Glu Phe Gly Leu Asp Ser Val Glu Leu Ala His
                100             105                 110

tat gat gtc ctg ttg tcc tac cca aat aag act cat ccc aac tac atc    384
Tyr Asp Val Leu Leu Ser Tyr Pro Asn Lys Thr His Pro Asn Tyr Ile
        115             120                 125

tca ata att aat gaa gat gga aat gag att ttc aac aca tca tta ttt    432
Ser Ile Ile Asn Glu Asp Gly Asn Glu Ile Phe Asn Thr Ser Leu Phe
        130             135                 140

gaa cca cct cct cca gga tat gaa aat gtt tcg gat att gta cca cct    480
Glu Pro Pro Pro Pro Gly Tyr Glu Asn Val Ser Asp Ile Val Pro Pro
145                 150                 155                 160

ttc agt gct ttc tct cct caa gga atg cca gag ggc gat cta gtg tat    528
Phe Ser Ala Phe Ser Pro Gln Gly Met Pro Glu Gly Asp Leu Val Tyr
                165             170                 175

gtt aac tat gca cga act gaa gac ttc ttt aaa ttg gaa cgg gac atg    576
Val Asn Tyr Ala Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg Asp Met
                180             185                 190

aaa atc aat tgc tct ggg aaa att gta att gcc aga tat ggg aaa gtt    624
Lys Ile Asn Cys Ser Gly Lys Ile Val Ile Ala Arg Tyr Gly Lys Val
        195             200                 205

ttc aga gga aat aag gtt aaa aat gcc cag ctg gca ggg gcc aaa gga    672
Phe Arg Gly Asn Lys Val Lys Asn Ala Gln Leu Ala Gly Ala Lys Gly
        210             215                 220

gtc att ctc tac tcc gac cct gct gac tac ttt gct cct ggg gtg aag    720
Val Ile Leu Tyr Ser Asp Pro Ala Asp Tyr Phe Ala Pro Gly Val Lys
225                 230                 235                 240
```

3

```
tcc tat cca gat ggt tgg aat ctt cct gga ggt ggt gtc cag cgt gga      768
Ser Tyr Pro Asp Gly Trp Asn Leu Pro Gly Gly Gly Val Gln Arg Gly
            245             250                 255

aat atc cta aat ctg aat ggt gca gga gac cct ctc aca cca ggt tac      816
Asn Ile Leu Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro Gly Tyr
            260             265                 270

cca gca aat gaa tat gct tat agg cgt gga att gca gag gct gtt ggt      864
Pro Ala Asn Glu Tyr Ala Tyr Arg Arg Gly Ile Ala Glu Ala Val Gly
            275             280                 285

ctt cca agt att cct gtt cat cca att gga tac tat gat gca cag aag      912
Leu Pro Ser Ile Pro Val His Pro Ile Gly Tyr Tyr Asp Ala Gln Lys
    290             295                 300

ctc cta gaa aaa atg ggt ggc tca gca cca cca gat agc agc tgg aga      960
Leu Leu Glu Lys Met Gly Gly Ser Ala Pro Pro Asp Ser Ser Trp Arg
305             310                 315                 320

gga agt ctc aaa gtg ccc tac aat gtt gga cct ggc ttt act gga aac     1008
Gly Ser Leu Lys Val Pro Tyr Asn Val Gly Pro Gly Phe Thr Gly Asn
            325             330                 335

ttt tct aca caa aaa gtc aag atg cac atc cac tct acc aat gaa gtg     1056
Phe Ser Thr Gln Lys Val Lys Met His Ile His Ser Thr Asn Glu Val
            340             345                 350

aca aga att tac aat gtg ata ggt act ctc aga gga gca gtg gaa cca     1104
Thr Arg Ile Tyr Asn Val Ile Gly Thr Leu Arg Gly Ala Val Glu Pro
            355             360                 365

gac aga tat gtc att ctg gga ggt cac cgg gac tca tgg gtg ttt ggt     1152
Asp Arg Tyr Val Ile Leu Gly Gly His Arg Asp Ser Trp Val Phe Gly
    370             375                 380

ggt att gac cct cag agt gga gca gct gtt gtt cat gaa att gtg agg     1200
Gly Ile Asp Pro Gln Ser Gly Ala Ala Val Val His Glu Ile Val Arg
385             390                 395                 400

agc ttt gga aca ctg aaa aag gaa ggg tgg aga cct aga aga aca att     1248
Ser Phe Gly Thr Leu Lys Lys Glu Gly Trp Arg Pro Arg Arg Thr Ile
            405             410                 415

ttg ttt gca agc tgg gat gca gaa gaa ttt ggt ctt ctt ggt tct act     1296
Leu Phe Ala Ser Trp Asp Ala Glu Glu Phe Gly Leu Leu Gly Ser Thr
            420             425                 430

gag tgg gca gag gag aat tca aga ctc ctt caa gag cgt ggc gtg gct     1344
Glu Trp Ala Glu Glu Asn Ser Arg Leu Leu Gln Glu Arg Gly Val Ala
            435             440                 445
```

```
tat att aat gct gac tca tct ata gaa gga aac tac act ctg aga gtt     1392
Tyr Ile Asn Ala Asp Ser Ser Ile Glu Gly Asn Tyr Thr Leu Arg Val
    450             455             460

gat tgt aca ccg ctg atg tac agc ttg gta cac aac cta aca aaa gag     1440
Asp Cys Thr Pro Leu Met Tyr Ser Leu Val His Asn Leu Thr Lys Glu
465             470             475             480

ctg aaa agc cct gat gaa ggc ttt gaa ggc aaa tct ctt tat gaa agt     1488
Leu Lys Ser Pro Asp Glu Gly Phe Glu Gly Lys Ser Leu Tyr Glu Ser
                485             490             495

tgg act aaa aaa agt cct tcc cca gag ttc agt ggc atg ccc agg ata     1536
Trp Thr Lys Lys Ser Pro Ser Pro Glu Phe Ser Gly Met Pro Arg Ile
            500             505             510

agc aaa ttg gga tct gga aat gat ttt gag gtg ttc ttc caa cga ctt     1584
Ser Lys Leu Gly Ser Gly Asn Asp Phe Glu Val Phe Phe Gln Arg Leu
            515             520             525

gga att gct tca ggc aga gca cgg tat act aaa aat tgg gaa aca aac     1632
Gly Ile Ala Ser Gly Arg Ala Arg Tyr Thr Lys Asn Trp Glu Thr Asn
    530             535             540

aaa ttc agc ggc tat cca ctg tat cac agt gtc tat gaa aca tat gag     1680
Lys Phe Ser Gly Tyr Pro Leu Tyr His Ser Val Tyr Glu Thr Tyr Glu
545             550             555             560

ttg gtg gaa aag ttt tat gat cca atg ttt aaa tat cac ctc act gtg     1728
Leu Val Glu Lys Phe Tyr Asp Pro Met Phe Lys Tyr His Leu Thr Val
                565             570             575

gcc cag gtt cga gga ggg atg gtg ttt gag cta gcc aat tcc ata gtg     1776
Ala Gln Val Arg Gly Gly Met Val Phe Glu Leu Ala Asn Ser Ile Val
            580             585             590

ctc cct ttt gat tgt cga gat tat gct gta gtt tta aga aag tat gct     1824
Leu Pro Phe Asp Cys Arg Asp Tyr Ala Val Val Leu Arg Lys Tyr Ala
            595             600             605

gac aaa atc tac agt att tct atg aaa cat cca cag gaa atg aag aca     1872
Asp Lys Ile Tyr Ser Ile Ser Met Lys His Pro Gln Glu Met Lys Thr
    610             615             620

tac agt gta tca ttt gat tca ctt ttt tct gca gta aag aat ttt aca     1920
Tyr Ser Val Ser Phe Asp Ser Leu Phe Ser Ala Val Lys Asn Phe Thr
625             630             635             640

gaa att gct tcc aag ttc agt gag aga ctc cag gac ttt gac aaa agc     1968
Glu Ile Ala Ser Lys Phe Ser Glu Arg Leu Gln Asp Phe Asp Lys Ser
                645             650             655
```

```
aac cca ata gta tta aga atg atg aat gat caa ctc atg ttt ctg gaa     2016
Asn Pro Ile Val Leu Arg Met Met Asn Asp Gln Leu Met Phe Leu Glu
            660                 665                 670

aga gca ttt att gat cca tta ggg tta cca gac agg cct ttt tat agg     2064
Arg Ala Phe Ile Asp Pro Leu Gly Leu Pro Asp Arg Pro Phe Tyr Arg
        675                 680                 685

cat gtc atc tat gct cca agc agc cac aac aag tat gca ggg gag tca     2112
His Val Ile Tyr Ala Pro Ser Ser His Asn Lys Tyr Ala Gly Glu Ser
        690                 695                 700

ttc cca gga att tat gat gct ctg ttt gat att gaa agc aaa gtg gac     2160
Phe Pro Gly Ile Tyr Asp Ala Leu Phe Asp Ile Glu Ser Lys Val Asp
705                 710                 715                 720

cct tcc aag gcc tgg gga gaa gtg aag aga cag att tat gtt gca gcc     2208
Pro Ser Lys Ala Trp Gly Glu Val Lys Arg Gln Ile Tyr Val Ala Ala
                725                 730                 735

ttc aca gtg cag gca gct gca gag act ttg agt gaa gta gcc taa         2253
Phe Thr Val Gln Ala Ala Ala Glu Thr Leu Ser Glu Val Ala
                740                 745                 750
```

<210> 2
<211> 750
<212> PRT
<213> Homo sapiens

<400> 2

```
Met Gly Asn Leu Leu His Glu Thr Asp Ser Ala Val Ala Thr Ala Arg
 1               5                  10                  15

Arg Pro Arg Trp Leu Cys Ala Gly Ala Leu Val Leu Ala Gly Gly Phe
            20                  25                  30

Phe Leu Leu Gly Phe Leu Phe Gly Trp Phe Ile Lys Ser Ser Asn Glu
        35                  40                  45

Ala Thr Asn Ile Thr Pro Lys His Asn Met Lys Ala Phe Leu Asp Glu
        50                  55                  60

Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr Gln Ile
65                  70                  75                  80

Pro His Leu Ala Gly Thr Glu Gln Asn Phe Gln Leu Ala Lys Gln Ile
                85                  90                  95

Gln Ser Gln Trp Lys Glu Phe Gly Leu Asp Ser Val Glu Leu Ala His
            100                 105                 110
```

```
Tyr Asp Val Leu Leu Ser Tyr Pro Asn Lys Thr His Pro Asn Tyr Ile
        115             120             125

Ser Ile Ile Asn Glu Asp Gly Asn Glu Ile Phe Asn Thr Ser Leu Phe
    130             135             140

Glu Pro Pro Pro Pro Gly Tyr Glu Asn Val Ser Asp Ile Val Pro Pro
145             150             155             160

Phe Ser Ala Phe Ser Pro Gln Gly Met Pro Glu Gly Asp Leu Val Tyr
                165             170             175

Val Asn Tyr Ala Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg Asp Met
            180             185             190

Lys Ile Asn Cys Ser Gly Lys Ile Val Ile Ala Arg Tyr Gly Lys Val
        195             200             205

Phe Arg Gly Asn Lys Val Lys Asn Ala Gln Leu Ala Gly Ala Lys Gly
    210             215             220

Val Ile Leu Tyr Ser Asp Pro Ala Asp Tyr Phe Ala Pro Gly Val Lys
225             230             235             240

Ser Tyr Pro Asp Gly Trp Asn Leu Pro Gly Gly Gly Val Gln Arg Gly
            245             250             255

Asn Ile Leu Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro Gly Tyr
        260             265             270

Pro Ala Asn Glu Tyr Ala Tyr Arg Arg Gly Ile Ala Glu Ala Val Gly
    275             280             285

Leu Pro Ser Ile Pro Val His Pro Ile Gly Tyr Tyr Asp Ala Gln Lys
    290             295             300

Leu Leu Glu Lys Met Gly Gly Ser Ala Pro Pro Asp Ser Ser Trp Arg
305             310             315             320

Gly Ser Leu Lys Val Pro Tyr Asn Val Gly Pro Gly Phe Thr Gly Asn
            325             330             335

Phe Ser Thr Gln Lys Val Lys Met His Ile His Ser Thr Asn Glu Val
            340             345             350

Thr Arg Ile Tyr Asn Val Ile Gly Thr Leu Arg Gly Ala Val Glu Pro
        355             360             365

Asp Arg Tyr Val Ile Leu Gly Gly His Arg Asp Ser Trp Val Phe Gly
    370             375             380

Gly Ile Asp Pro Gln Ser Gly Ala Ala Val Val His Glu Ile Val Arg
385             390             395             400
```

```
Ser Phe Gly Thr Leu Lys Lys Glu Gly Trp Arg Pro Arg Arg Thr Ile
            405             410                     415

Leu Phe Ala Ser Trp Asp Ala Glu Glu Phe Gly Leu Leu Gly Ser Thr
            420             425                     430

Glu Trp Ala Glu Glu Asn Ser Arg Leu Leu Gln Glu Arg Gly Val Ala
            435             440                     445

Tyr Ile Asn Ala Asp Ser Ser Ile Glu Gly Asn Tyr Thr Leu Arg Val
    450             455                     460

Asp Cys Thr Pro Leu Met Tyr Ser Leu Val His Asn Leu Thr Lys Glu
465             470             475                     480

Leu Lys Ser Pro Asp Glu Gly Phe Glu Gly Lys Ser Leu Tyr Glu Ser
            485             490                     495

Trp Thr Lys Lys Ser Pro Ser Pro Glu Phe Ser Gly Met Pro Arg Ile
            500             505                     510

Ser Lys Leu Gly Ser Gly Asn Asp Phe Glu Val Phe Phe Gln Arg Leu
            515             520                     525

Gly Ile Ala Ser Gly Arg Ala Arg Tyr Thr Lys Asn Trp Glu Thr Asn
    530             535                     540

Lys Phe Ser Gly Tyr Pro Leu Tyr His Ser Val Tyr Glu Thr Tyr Glu
545             550             555                     560

Leu Val Glu Lys Phe Tyr Asp Pro Met Phe Lys Tyr His Leu Thr Val
            565             570                     575

Ala Gln Val Arg Gly Gly Met Val Phe Glu Leu Ala Asn Ser Ile Val
            580             585                     590

Leu Pro Phe Asp Cys Arg Asp Tyr Ala Val Val Leu Arg Lys Tyr Ala
            595             600                     605

Asp Lys Ile Tyr Ser Ile Ser Met Lys His Pro Gln Glu Met Lys Thr
    610             615                     620

Tyr Ser Val Ser Phe Asp Ser Leu Phe Ser Ala Val Lys Asn Phe Thr
625             630             635                     640

Glu Ile Ala Ser Lys Phe Ser Glu Arg Leu Gln Asp Phe Asp Lys Ser
            645             650                     655

Asn Pro Ile Val Leu Arg Met Met Asn Asp Gln Leu Met Phe Leu Glu
            660             665                     670

Arg Ala Phe Ile Asp Pro Leu Gly Leu Pro Asp Arg Pro Phe Tyr Arg
            675             680                     685
```

8

```
His Val Ile Tyr Ala Pro Ser Ser His Asn Lys Tyr Ala Gly Glu Ser
    690             695             700
```

```
Phe Pro Gly Ile Tyr Asp Ala Leu Phe Asp Ile Glu Ser Lys Val Asp
705             710             715             720
```

```
Pro Ser Lys Ala Trp Gly Glu Val Lys Arg Gln Ile Tyr Val Ala Ala
            725             730             735
```

```
Phe Thr Val Gln Ala Ala Ala Glu Thr Leu Ser Glu Val Ala
            740             745             750
```

```
<210> 3
<211> 3768
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(3768)

<400> 3
atg gag ctg gcg gcc ttg tgc cgc tgg ggg ctc ctc ctc gcc ctc ttg    48
Met Glu Leu Ala Ala Leu Cys Arg Trp Gly Leu Leu Leu Ala Leu Leu
            -20             -15             -10

ccc ccc gga gcc gcg agc acc caa gtg tgc acc ggc aca gac atg aag    96
Pro Pro Gly Ala Ala Ser Thr Gln Val Cys Thr Gly Thr Asp Met Lys
        -5              -1   1               5

ctg cgg ctc cct gcc agt ccc gag acc cac ctg gac atg ctc cgc cac   144
Leu Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His
 10              15              20              25

ctc tac cag ggc tgc cag gtg gtg cag gga aac ctg gaa ctc acc tac   192
Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr
                30              35              40

ctg ccc acc aat gcc agc ctg tcc ttc ctg cag gat atc cag gag gtg   240
Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val
            45              50              55

cag ggc tac gtg ctc atc gct cac aac caa gtg agg cag gtc cca ctg   288
Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu
            60              65              70

cag agg ctg cgg att gtg cga ggc acc cag ctc ttt gag gac aac tat   336
Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
    75              80              85
```

9

```
gcc ctg gcc gtg cta gac aat gga gac ccg ctg aac aat acc acc cct    384
Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro
 90              95              100             105

gtc aca ggg gcc tcc cca gga ggc ctg cgg gag ctg cag ctt cga agc    432
Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser
                110             115             120

ctc aca gag atc ttg aaa gga ggg gtc ttg atc cag cgg aac ccc cag    480
Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
            125             130             135

ctc tgc tac cag gac acg att ttg tgg aag gac atc ttc cac aag aac    528
Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
        140             145             150

aac cag ctg gct ctc aca ctg ata gac acc aac cgc tct cgg gcc tgc    576
Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys
        155             160             165

cac ccc tgt tct ccg atg tgt aag ggc tcc cgc tgc tgg gga gag agt    624
His Pro Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser
170             175             180             185

tct gag gat tgt cag agc ctg acg cgc act gtc tgt gcc ggt ggc tgt    672
Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys
                190             195             200

gcc cgc tgc aag ggg cca ctg ccc act gac tgc tgc cat gag cag tgt    720
Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys
            205             210             215

gct gcc ggc tgc acg ggc ccc aag cac tct gac tgc ctg gcc tgc ctc    768
Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys Leu
            220             225             230

cac ttc aac cac agt ggc atc tgt gag ctg cac tgc cca gcc ctg gtc    816
His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu Val
        235             240             245

acc tac aac aca gac acg ttt gag tcc atg ccc aat ccc gag ggc cgg    864
Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg
250             255             260             265

tat aca ttc ggc gcc agc tgt gtg act gcc tgt ccc tac aac tac ctt    912
Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu
                270             275             280

tct acg gac gtg gga tcc tgc acc ctc gtc tgc ccc ctg cac aac caa    960
Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln
            285             290             295
```

10

```
gag gtg aca gca gag gat gga aca cag cgg tgt gag aag tgc agc aag     1008
Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys
        300             305             310

ccc tgt gcc cga gtg tgc tat ggt ctg ggc atg gag cac ttg cga gag     1056
Pro Cys Ala Arg Val Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu
        315             320             325

gtg agg gca gtt acc agt gcc aat atc cag gag ttt gct ggc tgc aag     1104
Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys
330             335             340             345

aag atc ttt ggg agc ctg gca ttt ctg ccg gag agc ttt gat ggg gac     1152
Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp
            350             355             360

cca gcc tcc aac act gcc ccg ctc cag cca gag cag ctc caa gtg ttt     1200
Pro Ala Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe
            365             370             375

gag act ctg gaa gag atc aca ggt tac cta tac atc tca gca tgg ccg     1248
Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro
        380             385             390

gac agc ctg cct gac ctc agc gtc ttc cag aac ctg caa gta atc cgg     1296
Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg
        395             400             405

gga cga att ctg cac aat ggc gcc tac tcg ctg acc ctg caa ggg ctg     1344
Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln Gly Leu
410             415             420             425

ggc atc agc tgg ctg ggg ctg cgc tca ctg agg gaa ctg ggc agt gga     1392
Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly Ser Gly
            430             435             440

ctg gcc ctc atc cac cat aac acc cac ctc tgc ttc gtg cac acg gtg     1440
Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe Val His Thr Val
            445             450             455

ccc tgg gac cag ctc ttt cgg aac ccg cac caa gct ctg ctc cac act     1488
Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln Ala Leu Leu His Thr
            460             465             470

gcc aac cgg cca gag gac gag tgt gtg ggc gag ggc ctg gcc tgc cac     1536
Ala Asn Arg Pro Glu Asp Glu Cys Val Gly Glu Gly Leu Ala Cys His
            475             480             485

cag ctg tgc gcc cga ggg cac tgc tgg ggt cca ggg ccc acc cag tgt     1584
Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro Thr Gln Cys
490             495             500             505
```

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gtc aac tgc agc cag ttc ctt cgg ggc cag gag tgc gtg gag gaa tgc    1632
Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys Val Glu Glu Cys
                510                 515                 520

cga gta ctg cag ggg ctc ccc agg gag tat gtg aat gcc agg cac tgt    1680
Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val Asn Ala Arg His Cys
                525                 530                 535

ttg ccg tgc cac cct gag tgt cag ccc cag aat ggc tca gtg acc tgt    1728
Leu Pro Cys His Pro Glu Cys Gln Pro Gln Asn Gly Ser Val Thr Cys
                540                 545                 550

ttt gga ccg gag gct gac cag tgt gtg gcc tgt gcc cac tat aag gac    1776
Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr Lys Asp
                555                 560                 565

cct ccc ttc tgc gtg gcc cgc tgc ccc agc ggt gtg aaa cct gac ctc    1824
Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys Pro Asp Leu
570                 575                 580                 585

tcc tac atg ccc atc tgg aag ttt cca gat gag gag ggc gca tgc cag    1872
Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu Gly Ala Cys Gln
                590                 595                 600

cct tgc ccc atc aac tgc acc cac tcc tgt gtg gac ctg gat gac aag    1920
Pro Cys Pro Ile Asn Cys Thr His Ser Cys Val Asp Leu Asp Asp Lys
                605                 610                 615

ggc tgc ccc gcc gag cag aga gcc agc cct ctg acg tcc atc gtc tct    1968
Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile Val Ser
                620                 625                 630

gcg gtg gtt ggc att ctg ctg gtc gtg gtc ttg ggg gtg gtc ttt ggg    2016
Ala Val Val Gly Ile Leu Leu Val Val Val Leu Gly Val Val Phe Gly
                635                 640                 645

atc ctc atc aag cga cgg cag cag aag atc cgg aag tac acg atg cgg    2064
Ile Leu Ile Lys Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr Met Arg
650                 655                 660                 665

aga ctg ctg cag gaa acg gag ctg gtg gag ccg ctg aca cct agc gga    2112
Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser Gly
                670                 675                 680

gcg atg ccc aac cag gcg cag atg cgg atc ctg aaa gag acg gag ctg    2160
Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr Glu Leu
                685                 690                 695

agg aag gtg aag gtg ctt gga tct ggc gct ttt ggc aca gtc tac aag    2208
Arg Lys Val Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys
                700                 705                 710
```

12

```
ggc atc tgg atc cct gat ggg gag aat gtg aaa att cca gtg gcc atc   2256
Gly Ile Trp Ile Pro Asp Gly Glu Asn Val Lys Ile Pro Val Ala Ile
    715             720             725

aaa gtg ttg agg gaa aac aca tcc ccc aaa gcc aac aaa gaa atc tta   2304
Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu
730             735             740             745

gac gaa gca tac gtg atg gct ggt gtg ggc tcc cca tat gtc tcc cgc   2352
Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg
                750             755             760

ctt ctg ggc atc tgc ctg aca tcc acg gtg cag ctg gtg aca cag ctt   2400
Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu
            765             770             775

atg ccc tat ggc tgc ctc tta gac cat gtc cgg gaa aac cgc gga cgc   2448
Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg
            780             785             790

ctg ggc tcc cag gac ctg ctg aac tgg tgt atg cag att gcc aag ggg   2496
Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly
    795             800             805

atg agc tac ctg gag gat gtg cgg ctc gta cac agg gac ttg gcc gct   2544
Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu Ala Ala
810             815             820             825

cgg aac gtg ctg gtc aag agt ccc aac cat gtc aaa att aca gac ttc   2592
Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe
            830             835             840

ggg ctg gct cgg ctg ctg gac att gac gag aca gag tac cat gca gat   2640
Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp
            845             850             855

ggg ggc aag gtg ccc atc aag tgg atg gcg ctg gag tcc att ctc cgc   2688
Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu Arg
            860             865             870

cgg cgg ttc acc cac cag agt gat gtg tgg agt tat ggt gtg act gtg   2736
Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val
    875             880             885

tgg gag ctg atg act ttt ggg gcc aaa cct tac gat ggg atc cca gcc   2784
Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro Ala
890             895             900             905

cgg gag atc cct gac ctg ctg gaa aag ggg gag cgg ctg ccc cag ccc   2832
Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro
            910             915             920
```

```
ccc atc tgc acc att gat gtc tac atg atc atg gtc aaa tgt tgg atg    2880
Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met
            925                     930                 935

att gac tct gaa tgt cgg cca aga ttc cgg gag ttg gtg tct gaa ttc    2928
Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser Glu Phe
            940                     945                 950

tcc cgc atg gcc agg gac ccc cag cgc ttt gtg gtc atc cag aat gag    2976
Ser Arg Met Ala Arg Asp Pro Gln Arg Phe Val Val Ile Gln Asn Glu
            955                     960                 965

gac ttg ggc cca gcc agt ccc ttg gac agc acc ttc tac cgc tca ctg    3024
Asp Leu Gly Pro Ala Ser Pro Leu Asp Ser Thr Phe Tyr Arg Ser Leu
970                     975                 980                 985

ctg gag gac gat gac atg ggg gac ctg gtg gat gct gag gag tat ctg    3072
Leu Glu Asp Asp Asp Met Gly Asp Leu Val Asp Ala Glu Glu Tyr Leu
                    990                     995                 1000

gta ccc cag cag ggc ttc ttc tgt cca gac cct gcc ccg ggc gct ggg    3120
Val Pro Gln Gln Gly Phe Phe Cys Pro Asp Pro Ala Pro Gly Ala Gly
                1005                    1010                1015

ggc atg gtc cac cac agg cac cgc agc tca tct acc agg agt ggc ggt    3168
Gly Met Val His His Arg His Arg Ser Ser Ser Thr Arg Ser Gly Gly
                1020                    1025                1030

ggg gac ctg aca cta ggg ctg gag ccc tct gaa gag gag gcc ccc agg    3216
Gly Asp Leu Thr Leu Gly Leu Glu Pro Ser Glu Glu Glu Ala Pro Arg
                1035                    1040                1045

tct cca ctg gca ccc tcc gaa ggg gct ggc tcc gat gta ttt gat ggt    3264
Ser Pro Leu Ala Pro Ser Glu Gly Ala Gly Ser Asp Val Phe Asp Gly
1050                    1055                1060                1065

gac ctg gga atg ggg gca gcc aag ggg ctg caa agc ctc ccc aca cat    3312
Asp Leu Gly Met Gly Ala Ala Lys Gly Leu Gln Ser Leu Pro Thr His
                    1070                    1075                1080

gac ccc agc cct cta cag cgg tac agt gag gac ccc aca gta ccc ctg    3360
Asp Pro Ser Pro Leu Gln Arg Tyr Ser Glu Asp Pro Thr Val Pro Leu
                1085                    1090                1095

ccc tct gag act gat ggc tac gtt gcc ccc ctg acc tgc agc ccc cag    3408
Pro Ser Glu Thr Asp Gly Tyr Val Ala Pro Leu Thr Cys Ser Pro Gln
                1100                    1105                1110

cct gaa tat gtg aac cag cca gat gtt cgg ccc cag ccc cct tcg ccc    3456
Pro Glu Tyr Val Asn Gln Pro Asp Val Arg Pro Gln Pro Pro Ser Pro
                1115                    1120                1125
```

14

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cga gag ggc cct ctg cct gct gcc cga cct gct ggt gcc act ctg gaa   3504
Arg Glu Gly Pro Leu Pro Ala Ala Arg Pro Ala Gly Ala Thr Leu Glu
1130               1135                1140                1145

agg gcc aag act ctc tcc cca ggg aag aat ggg gtc gtc aaa gac gtt   3552
Arg Ala Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp Val
            1150                1155                1160

ttt gcc ttt ggg ggt gcc gtg gag aac ccc gag tac ttg aca ccc cag   3600
Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr Pro Gln
            1165                1170                1175

gga gga gct gcc cct cag ccc cac cct cct cct gcc ttc agc cca gcc   3648
Gly Gly Ala Ala Pro Gln Pro His Pro Pro Pro Ala Phe Ser Pro Ala
            1180                1185                1190

ttc gac aac ctc tat tac tgg gac cag gac cca cca gag cgg ggg gct   3696
Phe Asp Asn Leu Tyr Tyr Trp Asp Gln Asp Pro Pro Glu Arg Gly Ala
    1195                1200                1205

cca ccc agc acc ttc aaa ggg aca cct acg gca gag aac cca gag tac   3744
Pro Pro Ser Thr Phe Lys Gly Thr Pro Thr Ala Glu Asn Pro Glu Tyr
1210                1215                1220                1225

ctg ggt ctg gac gtg cca gtg tga                                   3768
Leu Gly Leu Asp Val Pro Val
            1230
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<210> 4
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<212> PRT
<213> Homo sapiens

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Met Glu Leu Ala Ala Leu Cys Arg Trp Gly Leu Leu Leu Ala Leu Leu
 1               5                10                15

Pro Pro Gly Ala Ala Ser Thr Gln Val Cys Thr Gly Thr Asp Met Lys
            20                25                30

Leu Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His
        35                40                45

Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr
    50                55                60

Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val
65                70                75                80

Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu
            85                90                95
```

15

```
Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
            100                 105             110

Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro
            115                 120             125

Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser
    130             135             140

Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
145             150             155             160

Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
            165                 170             175

Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys
            180                 185             190

His Pro Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser
            195                 200             205

Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys
    210             215             220

Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys
225             230             235             240

Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys Leu
            245                 250             255

His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu Val
            260                 265             270

Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg
            275                 280             285

Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu
    290             295             300

Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln
305             310             315             320

Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys
            325                 330             335

Pro Cys Ala Arg Val Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu
            340                 345             350

Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys
            355                 360             365

Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp
    370             375             380
```

Pro Ala Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe
385   390   395   400

Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro
                405   410   415

Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg
            420   425   430

Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln Gly Leu
            435   440   445

Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly Ser Gly
    450   455   460

Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe Val His Thr Val
465   470   475   480

Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln Ala Leu Leu His Thr
                485   490   495

Ala Asn Arg Pro Glu Asp Glu Cys Val Gly Glu Gly Leu Ala Cys His
            500   505   510

Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro Thr Gln Cys
        515   520   525

Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys Val Glu Glu Cys
    530   535   540

Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val Asn Ala Arg His Cys
545   550   555   560

Leu Pro Cys His Pro Glu Cys Gln Pro Gln Asn Gly Ser Val Thr Cys
                565   570   575

Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr Lys Asp
            580   585   590

Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys Pro Asp Leu
        595   600   605

Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu Gly Ala Cys Gln
    610   615   620

Pro Cys Pro Ile Asn Cys Thr His Ser Cys Val Asp Leu Asp Asp Lys
625   630   635   640

Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile Val Ser
            645   650   655

Ala Val Val Gly Ile Leu Leu Val Val Val Leu Gly Val Val Phe Gly
        660   665   670

17

```
Ile Leu Ile Lys Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr Met Arg
        675                 680                 685

Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser Gly
        690                 695                 700

Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr Glu Leu
705                 710                 715                 720

Arg Lys Val Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys
                725                 730                 735

Gly Ile Trp Ile Pro Asp Gly Glu Asn Val Lys Ile Pro Val Ala Ile
            740                 745                 750

Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu
        755                 760                 765

Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg
        770                 775                 780

Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu
785                 790                 795                 800

Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg
                805                 810                 815

Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly
            820                 825                 830

Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu Ala Ala
            835                 840                 845

Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe
        850                 855                 860

Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp
865                 870                 875                 880

Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu Arg
                885                 890                 895

Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val
            900                 905                 910

Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro Ala
            915                 920                 925

Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro
        930                 935                 940

Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met
945                 950                 955                 960
```

18

Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser Glu Phe
                965   970   975

Ser Arg Met Ala Arg Asp Pro Gln Arg Phe Val Val Ile Gln Asn Glu
            980   985   990

Asp Leu Gly Pro Ala Ser Pro Leu Asp Ser Thr Phe Tyr Arg Ser Leu
        995   1000   1005

Leu Glu Asp Asp Asp Met Gly Asp Leu Val Asp Ala Glu Glu Tyr Leu
    1010   1015   1020

Val Pro Gln Gln Gly Phe Phe Cys Pro Asp Pro Ala Pro Gly Ala Gly
025   1030   1035   1040

Gly Met Val His His Arg His Arg Ser Ser Ser Thr Arg Ser Gly Gly
            1045   1050   1055

Gly Asp Leu Thr Leu Gly Leu Glu Pro Ser Glu Glu Glu Ala Pro Arg
        1060   1065   1070

Ser Pro Leu Ala Pro Ser Glu Gly Ala Gly Ser Asp Val Phe Asp Gly
        1075   1080   1085

Asp Leu Gly Met Gly Ala Ala Lys Gly Leu Gln Ser Leu Pro Thr His
    1090   1095   1100

Asp Pro Ser Pro Leu Gln Arg Tyr Ser Glu Asp Pro Thr Val Pro Leu
105   1110   1115   1120

Pro Ser Glu Thr Asp Gly Tyr Val Ala Pro Leu Thr Cys Ser Pro Gln
            1125   1130   1135

Pro Glu Tyr Val Asn Gln Pro Asp Val Arg Pro Gln Pro Pro Ser Pro
        1140   1145   1150

Arg Glu Gly Pro Leu Pro Ala Ala Arg Pro Ala Gly Ala Thr Leu Glu
        1155   1160   1165

Arg Ala Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp Val
    1170   1175   1180

Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr Pro Gln
185   1190   1195   1200

Gly Gly Ala Ala Pro Gln Pro His Pro Pro Pro Ala Phe Ser Pro Ala
            1205   1210   1215

Phe Asp Asn Leu Tyr Tyr Trp Asp Gln Asp Pro Pro Glu Arg Gly Ala
        1220   1225   1230

Pro Pro Ser Thr Phe Lys Gly Thr Pro Thr Ala Glu Asn Pro Glu Tyr
        1235   1240   1245

Leu Gly Leu Asp Val Pro Val
   1250   1255

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<212> DNA
<213> Homo sapiens

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<221> CDS
<222> (1)..(648)

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Met Gly Ser Pro Arg Ser Ala Leu Ser Cys Leu Leu Leu His Leu Leu
  1   5   10   15

gtt ctc tgc ctc caa gcc cag gta act gtt cag tcc tca cct aat ttt   96
Val Leu Cys Leu Gln Ala Gln Val Thr Val Gln Ser Ser Pro Asn Phe
               20   25   30

aca cag cat gtg agg gag cag agc ctg gtg acg gat cag ctc agc cgc   144
Thr Gln His Val Arg Glu Gln Ser Leu Val Thr Asp Gln Leu Ser Arg
           35   40   45

cgc ctc atc cgg acc tac cag ctc tac agc cgc acc agc ggg aag cac   192
Arg Leu Ile Arg Thr Tyr Gln Leu Tyr Ser Arg Thr Ser Gly Lys His
       50   55   60

gtg cag gtc ctg gcc aac aag cgc atc aac gcc atg gca gaa gac gga   240
Val Gln Val Leu Ala Asn Lys Arg Ile Asn Ala Met Ala Glu Asp Gly
 65   70   75   80

gac ccc ttc gcg aag ctc att gtg gag acc gat act ttt gga agc aga   288
Asp Pro Phe Ala Lys Leu Ile Val Glu Thr Asp Thr Phe Gly Ser Arg
                   85   90   95

gtc cga gtt cgc ggc gca gag aca ggt ctc tac atc tgc atg aac aag   336
Val Arg Val Arg Gly Ala Glu Thr Gly Leu Tyr Ile Cys Met Asn Lys
               100   105   110

aag ggg aag cta att gcc aag agc aac ggc aaa ggc aag gac tgc gta   384
Lys Gly Lys Leu Ile Ala Lys Ser Asn Gly Lys Gly Lys Asp Cys Val
           115   120   125

ttc aca gag atc gtg ctg gag aac aac tac acg gcg ctg cag aac gcc   432
Phe Thr Glu Ile Val Leu Glu Asn Asn Tyr Thr Ala Leu Gln Asn Ala
       130   135   140

```
aag tac gag ggc tgg tac atg gcc ttt acc cgc aag ggc cgg ccc cgc      480
Lys Tyr Glu Gly Trp Tyr Met Ala Phe Thr Arg Lys Gly Arg Pro Arg
145                 150                 155                 160

aag ggc tcc aag acg cgc cag cat cag cgc gag gtg cac ttc atg aag      528
Lys Gly Ser Lys Thr Arg Gln His Gln Arg Glu Val His Phe Met Lys
                165                 170                 175

cgc ctg ccg cgg ggc cac cac acc acc gag cag agc ctg cgc ttc gag      576
Arg Leu Pro Arg Gly His His Thr Thr Glu Gln Ser Leu Arg Phe Glu
            180                 185                 190

ttc ctc aac tac ccg ccc ttc acg cgc agc ctg cgc ggc agc cag agg      624
Phe Leu Asn Tyr Pro Pro Phe Thr Arg Ser Leu Arg Gly Ser Gln Arg
            195                 200                 205

act tgg gcc ccg gag ccc cga tag                                      648
Thr Trp Ala Pro Glu Pro Arg
        210                 215


<210> 6
<211> 215
<212> PRT
<213> Homo sapiens

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Met Gly Ser Pro Arg Ser Ala Leu Ser Cys Leu Leu Leu His Leu Leu
  1               5                  10                  15

Val Leu Cys Leu Gln Ala Gln Val Thr Val Gln Ser Ser Pro Asn Phe
            20                  25                  30

Thr Gln His Val Arg Glu Gln Ser Leu Val Thr Asp Gln Leu Ser Arg
            35                  40                  45

Arg Leu Ile Arg Thr Tyr Gln Leu Tyr Ser Arg Thr Ser Gly Lys His
        50                  55                  60

Val Gln Val Leu Ala Asn Lys Arg Ile Asn Ala Met Ala Glu Asp Gly
65                  70                  75                  80

Asp Pro Phe Ala Lys Leu Ile Val Glu Thr Asp Thr Phe Gly Ser Arg
                85                  90                  95

Val Arg Val Arg Gly Ala Glu Thr Gly Leu Tyr Ile Cys Met Asn Lys
            100                 105                 110

Lys Gly Lys Leu Ile Ala Lys Ser Asn Gly Lys Gly Lys Asp Cys Val
        115                 120                 125

Phe Thr Glu Ile Val Leu Glu Asn Asn Tyr Thr Ala Leu Gln Asn Ala
        130                 135                 140
```

21

```
Lys Tyr Glu Gly Trp Tyr Met Ala Phe Thr Arg Lys Gly Arg Pro Arg
145                 150             155             160

Lys Gly Ser Lys Thr Arg Gln His Gln Arg Glu Val His Phe Met Lys
                165             170             175

Arg Leu Pro Arg Gly His His Thr Thr Glu Gln Ser Leu Arg Phe Glu
            180             185             190

Phe Leu Asn Tyr Pro Pro Phe Thr Arg Ser Leu Arg Gly Ser Gln Arg
            195             200             205

Thr Trp Ala Pro Glu Pro Arg
    210             215


<210> 7
<211> 2256
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<213> Mus musculus

<220>
<221> CDS
<222> (1)..(2256)

<400> 7
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Met Trp Asn Ala Leu Gln Asp Arg Asp Ser Ala Glu Val Leu Gly His
  1               5                  10                  15

cgc cag cgc tgg ctc cgt gtt ggg aca ctg gtg ctg gct tta acc gga   96
Arg Gln Arg Trp Leu Arg Val Gly Thr Leu Val Leu Ala Leu Thr Gly
                20                  25                  30

acc ttc ctc att ggc ttc ctc ttt ggg tgg ttt ata aaa cct tcc aat  144
Thr Phe Leu Ile Gly Phe Leu Phe Gly Trp Phe Ile Lys Pro Ser Asn
            35                  40                  45

gaa gct act ggt aat gtt tcc cat tct ggc atg aag aag gag ttt ttg  192
Glu Ala Thr Gly Asn Val Ser His Ser Gly Met Lys Lys Glu Phe Leu
        50                  55                  60

cat gaa ttg aag gct gag aac atc aaa aaa ttt tta tac aat ttc aca  240
His Glu Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr
 65                 70                  75                  80

cgg aca cca cac ttg gca gga aca caa aat aat ttt gag ctt gca aag  288
Arg Thr Pro His Leu Ala Gly Thr Gln Asn Asn Phe Glu Leu Ala Lys
                85                  90                  95
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```
caa att cat gac cag tgg aaa gaa ttt ggc ctg gat ttg gtt gag tta    336
Gln Ile His Asp Gln Trp Lys Glu Phe Gly Leu Asp Leu Val Glu Leu
            100             105             110

tcc cat tac gat gtc ttg ctg tcc tat cca aat aaa act cat cct aac    384
Ser His Tyr Asp Val Leu Leu Ser Tyr Pro Asn Lys Thr His Pro Asn
            115             120             125

tat atc tca ata att aat gaa gat gga aat gag att ttc aaa aca tca    432
Tyr Ile Ser Ile Ile Asn Glu Asp Gly Asn Glu Ile Phe Lys Thr Ser
        130             135             140

tta tct gaa cag cca ccc cca gga tat gag aat ata tca gat gta gtg    480
Leu Ser Glu Gln Pro Pro Pro Gly Tyr Glu Asn Ile Ser Asp Val Val
145             150             155             160

cca cca tac agt gcc ttc tct cca caa ggg aca cca gag ggt gat cta    528
Pro Pro Tyr Ser Ala Phe Ser Pro Gln Gly Thr Pro Glu Gly Asp Leu
                165             170             175

gtg tat gtc aac tat gca cga act gaa gac ttc ttt aaa ctg gaa cgg    576
Val Tyr Val Asn Tyr Ala Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg
                180             185             190

gaa atg aag atc agt tgt tct ggg aag att gtg att gcc aga tat ggg    624
Glu Met Lys Ile Ser Cys Ser Gly Lys Ile Val Ile Ala Arg Tyr Gly
            195             200             205

aaa gtg ttc aga gga aat atg gtt aaa aat gct caa ctg gca ggg gca    672
Lys Val Phe Arg Gly Asn Met Val Lys Asn Ala Gln Leu Ala Gly Ala
            210             215             220

aaa gga atg att ctg tac tca gac cct gct gac tac ttt gtt cct gcg    720
Lys Gly Met Ile Leu Tyr Ser Asp Pro Ala Asp Tyr Phe Val Pro Ala
225             230             235             240

gtg aag tcc tat cca gat ggc tgg aac ctc cct gga ggt ggt gtc caa    768
Val Lys Ser Tyr Pro Asp Gly Trp Asn Leu Pro Gly Gly Gly Val Gln
                245             250             255

cgt gga aat gtc tta aat ctt aat ggt gca ggt gac ccg ctc aca cca    816
Arg Gly Asn Val Leu Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro
                260             265             270

ggt tac cca gca aat gaa cat gct tat agg cat gag ttg aca aac gct    864
Gly Tyr Pro Ala Asn Glu His Ala Tyr Arg His Glu Leu Thr Asn Ala
            275             280             285

gtt ggc ctt cca agt att cct gtc cat cct att gga tat gat gat gca    912
Val Gly Leu Pro Ser Ile Pro Val His Pro Ile Gly Tyr Asp Asp Ala
            290             295             300
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cag aaa ctc tta gaa cac atg ggt ggt cca gca ccc cct gac agt agc    960
Gln Lys Leu Leu Glu His Met Gly Gly Pro Ala Pro Pro Asp Ser Ser
305             310             315             320


tgg aag gga gga tta aaa gtg cct tac aac gtg gga cct ggc ttt gct   1008
Trp Lys Gly Gly Leu Lys Val Pro Tyr Asn Val Gly Pro Gly Phe Ala
            325             330             335


gga aac ttt tca aca caa aag gtc aag atg cat att cac tct tac act   1056
Gly Asn Phe Ser Thr Gln Lys Val Lys Met His Ile His Ser Tyr Thr
            340             345             350


aaa gtg aca aga atc tat aat gtc att ggc acc ctc aaa gga gct ctg   1104
Lys Val Thr Arg Ile Tyr Asn Val Ile Gly Thr Leu Lys Gly Ala Leu
            355             360             365


gaa cca gac aga tat gtt att ctt gga ggt cac cga gac gct tgg gta   1152
Glu Pro Asp Arg Tyr Val Ile Leu Gly Gly His Arg Asp Ala Trp Val
            370             375             380


ttt ggt ggc att gac cct cag agt gga gca gct gtt gtt cat gaa att   1200
Phe Gly Gly Ile Asp Pro Gln Ser Gly Ala Ala Val Val His Glu Ile
385             390             395             400


gtg cgg agc ttt gga acc ctg aag aag aaa gga cgg agg cct aga agg   1248
Val Arg Ser Phe Gly Thr Leu Lys Lys Lys Gly Arg Arg Pro Arg Arg
            405             410             415


aca att ttg ttt gca agc tgg gat gca gaa gaa ttt ggc ctt ctt ggt   1296
Thr Ile Leu Phe Ala Ser Trp Asp Ala Glu Glu Phe Gly Leu Leu Gly
            420             425             430


tct act gag tgg gca gag gaa cat tca aga ctc cta caa gag cga ggt   1344
Ser Thr Glu Trp Ala Glu Glu His Ser Arg Leu Leu Gln Glu Arg Gly
            435             440             445


gtg gct tat att aat gct gat tct tcc ata gaa gga aat tac act cta   1392
Val Ala Tyr Ile Asn Ala Asp Ser Ser Ile Glu Gly Asn Tyr Thr Leu
            450             455             460


aga gtt gat tgc aca cca ctg atg tac agc tta gtg tac aac cta aca   1440
Arg Val Asp Cys Thr Pro Leu Met Tyr Ser Leu Val Tyr Asn Leu Thr
465             470             475             480


aaa gag ctg caa agc cca gat gaa ggt ttt gaa gga aaa tct ctt tat   1488
Lys Glu Leu Gln Ser Pro Asp Glu Gly Phe Glu Gly Lys Ser Leu Tyr
            485             490             495


gac agc tgg aaa gaa aag agt cct tca cct gag ttc att gga atg ccc   1536
Asp Ser Trp Lys Glu Lys Ser Pro Ser Pro Glu Phe Ile Gly Met Pro
            500             505             510
```

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aga att agc aag ctg ggg tct ggc aat gat ttt gaa gtg ttc ttc caa    1584
Arg Ile Ser Lys Leu Gly Ser Gly Asn Asp Phe Glu Val Phe Phe Gln
        515             520             525

aga ctt gga att gct tca ggc aga gcc cga tat act aaa aat tgg aaa    1632
Arg Leu Gly Ile Ala Ser Gly Arg Ala Arg Tyr Thr Lys Asn Trp Lys
        530             535             540

act aac aaa gtc agc agc tat cct ctc tat cac agt gtc tat gaa aca    1680
Thr Asn Lys Val Ser Ser Tyr Pro Leu Tyr His Ser Val Tyr Glu Thr
545             550             555             560

tat gag ctg gta gta aaa ttt tat gac cca aca ttt aaa tac cac ctc    1728
Tyr Glu Leu Val Val Lys Phe Tyr Asp Pro Thr Phe Lys Tyr His Leu
                565             570             575

act gtg gcc cag gtt cga gga gcg atg gta ttt gaa ctt gcc aat tct    1776
Thr Val Ala Gln Val Arg Gly Ala Met Val Phe Glu Leu Ala Asn Ser
            580             585             590

ata gtg ctt ccc ttt gac tgc caa agt tat gct gta gct ctg aag aag    1824
Ile Val Leu Pro Phe Asp Cys Gln Ser Tyr Ala Val Ala Leu Lys Lys
            595             600             605

tat gct gac act atc tac aat att tca atg aaa cat cca caa gaa atg    1872
Tyr Ala Asp Thr Ile Tyr Asn Ile Ser Met Lys His Pro Gln Glu Met
        610             615             620

aag gct tac atg ata tca ttt gat tca ctg ttt tct gca gtc aat aat    1920
Lys Ala Tyr Met Ile Ser Phe Asp Ser Leu Phe Ser Ala Val Asn Asn
625             630             635             640

ttt aca gat gtt gca tct aag ttc aat cag aga ctg caa gag tta gac    1968
Phe Thr Asp Val Ala Ser Lys Phe Asn Gln Arg Leu Gln Glu Leu Asp
                645             650             655

aaa agc aac ccc ata tta ctg aga att atg aat gac cag ctg atg tat    2016
Lys Ser Asn Pro Ile Leu Leu Arg Ile Met Asn Asp Gln Leu Met Tyr
                660             665             670

ctg gaa cgt gca ttc att gat cct tta ggc tta cca gga agg cct ttc    2064
Leu Glu Arg Ala Phe Ile Asp Pro Leu Gly Leu Pro Gly Arg Pro Phe
                675             680             685

tac agg cat acc atc tat gct cca agc agc cac aac aag tat gca gga    2112
Tyr Arg His Thr Ile Tyr Ala Pro Ser Ser His Asn Lys Tyr Ala Gly
        690             695             700

gaa tca ttc cct ggg att tat gat gcc ctt ttt gat ata agt agc aaa    2160
Glu Ser Phe Pro Gly Ile Tyr Asp Ala Leu Phe Asp Ile Ser Ser Lys
705             710             715             720
```

```
gtc aat gct tct aag gcc tgg aac gaa gtg aag aga cag att tct att   2208
Val Asn Ala Ser Lys Ala Trp Asn Glu Val Lys Arg Gln Ile Ser Ile
            725             730             735

gca acc ttt aca gtg caa gct gca gca gag act ctg agg gaa gta gct   2256
Ala Thr Phe Thr Val Gln Ala Ala Ala Glu Thr Leu Arg Glu Val Ala
            740             745             750
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<210> 8
<211> 752
<212> PRT
<213> Mus musculus

<400> 8

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Met Trp Asn Ala Leu Gln Asp Arg Asp Ser Ala Glu Val Leu Gly His
  1               5                  10                  15

Arg Gln Arg Trp Leu Arg Val Gly Thr Leu Val Leu Ala Leu Thr Gly
             20                  25                  30

Thr Phe Leu Ile Gly Phe Leu Phe Gly Trp Phe Ile Lys Pro Ser Asn
         35                  40                  45

Glu Ala Thr Gly Asn Val Ser His Ser Gly Met Lys Lys Glu Phe Leu
         50                  55                  60

His Glu Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr
 65                  70                  75                  80

Arg Thr Pro His Leu Ala Gly Thr Gln Asn Asn Phe Glu Leu Ala Lys
                 85                  90                  95

Gln Ile His Asp Gln Trp Lys Glu Phe Gly Leu Asp Leu Val Glu Leu
                100                 105                 110

Ser His Tyr Asp Val Leu Leu Ser Tyr Pro Asn Lys Thr His Pro Asn
            115                 120                 125

Tyr Ile Ser Ile Ile Asn Glu Asp Gly Asn Glu Ile Phe Lys Thr Ser
        130                 135                 140

Leu Ser Glu Gln Pro Pro Pro Gly Tyr Glu Asn Ile Ser Asp Val Val
145                 150                 155                 160

Pro Pro Tyr Ser Ala Phe Ser Pro Gln Gly Thr Pro Glu Gly Asp Leu
                165                 170                 175

Val Tyr Val Asn Tyr Ala Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg
            180                 185                 190

Glu Met Lys Ile Ser Cys Ser Gly Lys Ile Val Ile Ala Arg Tyr Gly
            195                 200                 205
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26

```
Lys Val Phe Arg Gly Asn Met Val Lys Asn Ala Gln Leu Ala Gly Ala
    210                 215                 220

Lys Gly Met Ile Leu Tyr Ser Asp Pro Ala Asp Tyr Phe Val Pro Ala
225                 230                 235                 240

Val Lys Ser Tyr Pro Asp Gly Trp Asn Leu Pro Gly Gly Gly Val Gln
                245                 250                 255

Arg Gly Asn Val Leu Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro
            260                 265                 270

Gly Tyr Pro Ala Asn Glu His Ala Tyr Arg His Glu Leu Thr Asn Ala
        275                 280                 285

Val Gly Leu Pro Ser Ile Pro Val His Pro Ile Gly Tyr Asp Asp Ala
    290                 295                 300

Gln Lys Leu Leu Glu His Met Gly Gly Pro Ala Pro Pro Asp Ser Ser
305                 310                 315                 320

Trp Lys Gly Gly Leu Lys Val Pro Tyr Asn Val Gly Pro Gly Phe Ala
                325                 330                 335

Gly Asn Phe Ser Thr Gln Lys Val Lys Met His Ile His Ser Tyr Thr
            340                 345                 350

Lys Val Thr Arg Ile Tyr Asn Val Ile Gly Thr Leu Lys Gly Ala Leu
        355                 360                 365

Glu Pro Asp Arg Tyr Val Ile Leu Gly Gly His Arg Asp Ala Trp Val
    370                 375                 380

Phe Gly Gly Ile Asp Pro Gln Ser Gly Ala Ala Val Val His Glu Ile
385                 390                 395                 400

Val Arg Ser Phe Gly Thr Leu Lys Lys Lys Gly Arg Arg Pro Arg Arg
                405                 410                 415

Thr Ile Leu Phe Ala Ser Trp Asp Ala Glu Glu Phe Gly Leu Leu Gly
            420                 425                 430

Ser Thr Glu Trp Ala Glu Glu His Ser Arg Leu Leu Gln Glu Arg Gly
        435                 440                 445

Val Ala Tyr Ile Asn Ala Asp Ser Ser Ile Glu Gly Asn Tyr Thr Leu
    450                 455                 460

Arg Val Asp Cys Thr Pro Leu Met Tyr Ser Leu Val Tyr Asn Leu Thr
465                 470                 475                 480

Lys Glu Leu Gln Ser Pro Asp Glu Gly Phe Glu Gly Lys Ser Leu Tyr
                485                 490                 495
```

Asp Ser Trp Lys Glu Lys Ser Pro Ser Pro Glu Phe Ile Gly Met Pro
            500   505   510

Arg Ile Ser Lys Leu Gly Ser Gly Asn Asp Phe Glu Val Phe Phe Gln
            515   520   525

Arg Leu Gly Ile Ala Ser Gly Arg Ala Arg Tyr Thr Lys Asn Trp Lys
    530   535   540

Thr Asn Lys Val Ser Ser Tyr Pro Leu Tyr His Ser Val Tyr Glu Thr
545   550   555   560

Tyr Glu Leu Val Val Lys Phe Tyr Asp Pro Thr Phe Lys Tyr His Leu
                565   570   575

Thr Val Ala Gln Val Arg Gly Ala Met Val Phe Glu Leu Ala Asn Ser
            580   585   590

Ile Val Leu Pro Phe Asp Cys Gln Ser Tyr Ala Val Ala Leu Lys Lys
            595   600   605

Tyr Ala Asp Thr Ile Tyr Asn Ile Ser Met Lys His Pro Gln Glu Met
    610   615   620

Lys Ala Tyr Met Ile Ser Phe Asp Ser Leu Phe Ser Ala Val Asn Asn
625   630   635   640

Phe Thr Asp Val Ala Ser Lys Phe Asn Gln Arg Leu Gln Glu Leu Asp
            645   650   655

Lys Ser Asn Pro Ile Leu Leu Arg Ile Met Asn Asp Gln Leu Met Tyr
            660   665   670

Leu Glu Arg Ala Phe Ile Asp Pro Leu Gly Leu Pro Gly Arg Pro Phe
    675   680   685

Tyr Arg His Thr Ile Tyr Ala Pro Ser Ser His Asn Lys Tyr Ala Gly
    690   695   700

Glu Ser Phe Pro Gly Ile Tyr Asp Ala Leu Phe Asp Ile Ser Ser Lys
705   710   715   720

Val Asn Ala Ser Lys Ala Trp Asn Glu Val Lys Arg Gln Ile Ser Ile
            725   730   735

Ala Thr Phe Thr Val Gln Ala Ala Ala Glu Thr Leu Arg Glu Val Ala
            740   745   750

<210> 9
<211> 2082
<212> DNA

<213> Mus musculus

<220>
<221> CDS
<222> (1)..(2082)

<400> 9
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atg aag aag gag ttt ttg cat gaa ttg aag gct gag aac atc aaa aaa    48
Met Lys Lys Glu Phe Leu His Glu Leu Lys Ala Glu Asn Ile Lys Lys
 1               5                  10                  15

ttt tta tac aat ttc aca cgg aca cca cac ttg gca gga aca caa aat    96
Phe Leu Tyr Asn Phe Thr Arg Thr Pro His Leu Ala Gly Thr Gln Asn
            20                  25                  30

aat ttt gag ctt gca aag caa att cat gac cag tgg aaa gaa ttt ggc   144
Asn Phe Glu Leu Ala Lys Gln Ile His Asp Gln Trp Lys Glu Phe Gly
        35                  40                  45

ctg gat ttg gtt gag tta tcc cat tac gat gtc ttg ctg tcc tat cca   192
Leu Asp Leu Val Glu Leu Ser His Tyr Asp Val Leu Leu Ser Tyr Pro
    50                  55                  60

aat aaa act cat cct aac tat atc tca ata att aat gaa gat gga aat   240
Asn Lys Thr His Pro Asn Tyr Ile Ser Ile Ile Asn Glu Asp Gly Asn
 65                  70                  75                  80

gag att ttc aaa aca tca tta tct gaa cag cca ccc cca gga tat gag   288
Glu Ile Phe Lys Thr Ser Leu Ser Glu Gln Pro Pro Pro Gly Tyr Glu
                85                  90                  95

aat ata tca gat gta gtg cca cca tac agt gcc ttc tct cca caa ggg   336
Asn Ile Ser Asp Val Val Pro Pro Tyr Ser Ala Phe Ser Pro Gln Gly
            100                 105                 110

aca cca gag ggt gat cta gtg tat gtc aac tat gca cga act gaa gac   384
Thr Pro Glu Gly Asp Leu Val Tyr Val Asn Tyr Ala Arg Thr Glu Asp
            115                 120                 125

ttc ttt aaa ctg gaa cgg gaa atg aag atc agt tgt tct ggg aag att   432
Phe Phe Lys Leu Glu Arg Glu Met Lys Ile Ser Cys Ser Gly Lys Ile
    130                 135                 140

gtg att gcc aga tat ggg aaa gtg ttc aga gga aat atg gtt aaa aat   480
Val Ile Ala Arg Tyr Gly Lys Val Phe Arg Gly Asn Met Val Lys Asn
145                 150                 155                 160

gct caa ctg gca ggg gca aaa gga atg att ctg tac tca gac cct gct   528
Ala Gln Leu Ala Gly Ala Lys Gly Met Ile Leu Tyr Ser Asp Pro Ala
                165                 170                 175
```

```
gac tac ttt gtt cct gcg gtg aag tcc tat cca gat ggc tgg aac ctc    576
Asp Tyr Phe Val Pro Ala Val Lys Ser Tyr Pro Asp Gly Trp Asn Leu
            180                 185                 190

cct gga ggt ggt gtc caa cgt gga aat gtc tta aat ctt aat ggt gca    624
Pro Gly Gly Gly Val Gln Arg Gly Asn Val Leu Asn Leu Asn Gly Ala
            195                 200                 205

ggt gac ccg ctc aca cca ggt tac cca gca aat gaa cat gct tat agg    672
Gly Asp Pro Leu Thr Pro Gly Tyr Pro Ala Asn Glu His Ala Tyr Arg
            210                 215                 220

cat gag ttg aca aac gct gtt ggc ctt cca agt att cct gtc cat cct    720
His Glu Leu Thr Asn Ala Val Gly Leu Pro Ser Ile Pro Val His Pro
225                 230                 235                 240

att gga tat gat gat gca cag aaa ctc tta gaa cac atg ggt ggt cca    768
Ile Gly Tyr Asp Asp Ala Gln Lys Leu Leu Glu His Met Gly Gly Pro
                245                 250                 255

gca ccc cct gac agt agc tgg aag gga gga tta aaa gtg cct tac aac    816
Ala Pro Pro Asp Ser Ser Trp Lys Gly Gly Leu Lys Val Pro Tyr Asn
            260                 265                 270

gtg gga cct ggc ttt gct gga aac ttt tca aca caa aag gtc aag atg    864
Val Gly Pro Gly Phe Ala Gly Asn Phe Ser Thr Gln Lys Val Lys Met
            275                 280                 285

cat att cac tct tac act aaa gtg aca aga atc tat aat gtc att ggc    912
His Ile His Ser Tyr Thr Lys Val Thr Arg Ile Tyr Asn Val Ile Gly
            290                 295                 300

acc ctc aaa gga gct ctg gaa cca gac aga tat gtt att ctt gga ggt    960
Thr Leu Lys Gly Ala Leu Glu Pro Asp Arg Tyr Val Ile Leu Gly Gly
305                 310                 315                 320

cac cga gac gct tgg gta ttt ggt ggc att gac cct cag agt gga gca    1008
His Arg Asp Ala Trp Val Phe Gly Gly Ile Asp Pro Gln Ser Gly Ala
                325                 330                 335

gct gtt gtt cat gaa att gtg cgg agc ttt gga acc ctg aag aag aaa    1056
Ala Val Val His Glu Ile Val Arg Ser Phe Gly Thr Leu Lys Lys Lys
            340                 345                 350

gga cgg agg cct aga agg aca att ttg ttt gca agc tgg gat gca gaa    1104
Gly Arg Arg Pro Arg Arg Thr Ile Leu Phe Ala Ser Trp Asp Ala Glu
            355                 360                 365

gaa ttt ggc ctt ctt ggt tct act gag tgg gca gag gaa cat tca aga    1152
Glu Phe Gly Leu Leu Gly Ser Thr Glu Trp Ala Glu Glu His Ser Arg
            370                 375                 380
```

30

```
ctc cta caa gag cga ggt gtg gct tat att aat gct gat tct tcc ata   1200
Leu Leu Gln Glu Arg Gly Val Ala Tyr Ile Asn Ala Asp Ser Ser Ile
385             390             395                 400

gaa gga aat tac act cta aga gtt gat tgc aca cca ctg atg tac agc   1248
Glu Gly Asn Tyr Thr Leu Arg Val Asp Cys Thr Pro Leu Met Tyr Ser
                405             410                 415

tta gtg tac aac cta aca aaa gag ctg caa agc cca gat gaa ggt ttt   1296
Leu Val Tyr Asn Leu Thr Lys Glu Leu Gln Ser Pro Asp Glu Gly Phe
            420             425             430

gaa gga aaa tct ctt tat gac agc tgg aaa gaa aag agt cct tca cct   1344
Glu Gly Lys Ser Leu Tyr Asp Ser Trp Lys Glu Lys Ser Pro Ser Pro
            435             440             445

gag ttc att gga atg ccc aga att agc aag ctg ggg tct ggc aat gat   1392
Glu Phe Ile Gly Met Pro Arg Ile Ser Lys Leu Gly Ser Gly Asn Asp
            450             455             460

ttt gaa gtg ttc ttc caa aga ctt gga att gct tca ggc aga gcc cga   1440
Phe Glu Val Phe Phe Gln Arg Leu Gly Ile Ala Ser Gly Arg Ala Arg
465             470             475                 480

tat act aaa aat tgg aaa act aac aaa gtc agc agc tat cct ctc tat   1488
Tyr Thr Lys Asn Trp Lys Thr Asn Lys Val Ser Ser Tyr Pro Leu Tyr
                485             490                 495

cac agt gtc tat gaa aca tat gag ctg gta gta aaa ttt tat gac cca   1536
His Ser Val Tyr Glu Thr Tyr Glu Leu Val Val Lys Phe Tyr Asp Pro
            500             505             510

aca ttt aaa tac cac ctc act gtg gcc cag gtt cga gga gcg atg gta   1584
Thr Phe Lys Tyr His Leu Thr Val Ala Gln Val Arg Gly Ala Met Val
            515             520             525

ttt gaa ctt gcc aat tct ata gtg ctt ccc ttt gac tgc caa agt tat   1632
Phe Glu Leu Ala Asn Ser Ile Val Leu Pro Phe Asp Cys Gln Ser Tyr
            530             535             540

gct gta gct ctg aag aag tat gct gac act atc tac aat att tca atg   1680
Ala Val Ala Leu Lys Lys Tyr Ala Asp Thr Ile Tyr Asn Ile Ser Met
545             550             555                 560

aaa cat cca caa gaa atg aag gct tac atg ata tca ttt gat tca ctg   1728
Lys His Pro Gln Glu Met Lys Ala Tyr Met Ile Ser Phe Asp Ser Leu
                565             570                 575

ttt tct gca gtc aat aat ttt aca gat gtt gca tct aag ttc aat cag   1776
Phe Ser Ala Val Asn Asn Phe Thr Asp Val Ala Ser Lys Phe Asn Gln
            580             585             590
```

```
aga ctg caa gag tta gac aaa agc aac ccc ata tta ctg aga att atg    1824
Arg Leu Gln Glu Leu Asp Lys Ser Asn Pro Ile Leu Leu Arg Ile Met
        595                 600                 605

aat gac cag ctg atg tat ctg gaa cgt gca ttc att gat cct tta ggc    1872
Asn Asp Gln Leu Met Tyr Leu Glu Arg Ala Phe Ile Asp Pro Leu Gly
        610                 615                 620

tta cca gga agg cct ttc tac agg cat acc atc tat gct cca agc agc    1920
Leu Pro Gly Arg Pro Phe Tyr Arg His Thr Ile Tyr Ala Pro Ser Ser
625                 630                 635                 640

cac aac aag tat gca gga gaa tca ttc cct ggg att tat gat gcc ctt    1968
His Asn Lys Tyr Ala Gly Glu Ser Phe Pro Gly Ile Tyr Asp Ala Leu
                645                 650                 655

ttt gat ata agt agc aaa gtc aat gct tct aag gcc tgg aac gaa gtg    2016
Phe Asp Ile Ser Ser Lys Val Asn Ala Ser Lys Ala Trp Asn Glu Val
                660                 665                 670

aag aga cag att tct att gca acc ttt aca gtg caa gct gca gca gag    2064
Lys Arg Gln Ile Ser Ile Ala Thr Phe Thr Val Gln Ala Ala Ala Glu
        675                 680                 685

act ctg agg gaa gta gct                                            2082
Thr Leu Arg Glu Val Ala
        690
```

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<210> 10
<211> 694
<212> PRT
<213> Mus musculus

<400> 10
Met Lys Lys Glu Phe Leu His Glu Leu Lys Ala Glu Asn Ile Lys Lys
  1               5                  10                  15

Phe Leu Tyr Asn Phe Thr Arg Thr Pro His Leu Ala Gly Thr Gln Asn
              20                  25                  30

Asn Phe Glu Leu Ala Lys Gln Ile His Asp Gln Trp Lys Glu Phe Gly
          35                  40                  45

Leu Asp Leu Val Glu Leu Ser His Tyr Asp Val Leu Leu Ser Tyr Pro
      50                  55                  60

Asn Lys Thr His Pro Asn Tyr Ile Ser Ile Ile Asn Glu Asp Gly Asn
65                  70                  75                  80

Glu Ile Phe Lys Thr Ser Leu Ser Glu Gln Pro Pro Pro Gly Tyr Glu
                  85                  90                  95
```

32

Asn Ile Ser Asp Val Val Pro Pro Tyr Ser Ala Phe Ser Pro Gln Gly
            100   105   110

Thr Pro Glu Gly Asp Leu Val Tyr Val Asn Tyr Ala Arg Thr Glu Asp
            115   120   125

Phe Phe Lys Leu Glu Arg Glu Met Lys Ile Ser Cys Ser Gly Lys Ile
    130   135   140

Val Ile Ala Arg Tyr Gly Lys Val Phe Arg Gly Asn Met Val Lys Asn
145   150   155   160

Ala Gln Leu Ala Gly Ala Lys Gly Met Ile Leu Tyr Ser Asp Pro Ala
                165   170   175

Asp Tyr Phe Val Pro Ala Val Lys Ser Tyr Pro Asp Gly Trp Asn Leu
            180   185   190

Pro Gly Gly Gly Val Gln Arg Gly Asn Val Leu Asn Leu Asn Gly Ala
            195   200   205

Gly Asp Pro Leu Thr Pro Gly Tyr Pro Ala Asn Glu His Ala Tyr Arg
    210   215   220

His Glu Leu Thr Asn Ala Val Gly Leu Pro Ser Ile Pro Val His Pro
225   230   235   240

Ile Gly Tyr Asp Asp Ala Gln Lys Leu Leu Glu His Met Gly Gly Pro
            245   250   255

Ala Pro Pro Asp Ser Ser Trp Lys Gly Gly Leu Lys Val Pro Tyr Asn
            260   265   270

Val Gly Pro Gly Phe Ala Gly Asn Phe Ser Thr Gln Lys Val Lys Met
            275   280   285

His Ile His Ser Tyr Thr Lys Val Thr Arg Ile Tyr Asn Val Ile Gly
    290   295   300

Thr Leu Lys Gly Ala Leu Glu Pro Asp Arg Tyr Val Ile Leu Gly Gly
305   310   315   320

His Arg Asp Ala Trp Val Phe Gly Gly Ile Asp Pro Gln Ser Gly Ala
                325   330   335

Ala Val Val His Glu Ile Val Arg Ser Phe Gly Thr Leu Lys Lys Lys
            340   345   350

Gly Arg Arg Pro Arg Arg Thr Ile Leu Phe Ala Ser Trp Asp Ala Glu
    355   360   365

Glu Phe Gly Leu Leu Gly Ser Thr Glu Trp Ala Glu Glu His Ser Arg
    370   375   380

33

```
Leu Leu Gln Glu Arg Gly Val Ala Tyr Ile Asn Ala Asp Ser Ser Ile
385             390             395             400

Glu Gly Asn Tyr Thr Leu Arg Val Asp Cys Thr Pro Leu Met Tyr Ser
            405             410             415

Leu Val Tyr Asn Leu Thr Lys Glu Leu Gln Ser Pro Asp Glu Gly Phe
            420             425             430

Glu Gly Lys Ser Leu Tyr Asp Ser Trp Lys Glu Lys Ser Pro Ser Pro
            435             440             445

Glu Phe Ile Gly Met Pro Arg Ile Ser Lys Leu Gly Ser Gly Asn Asp
        450             455             460

Phe Glu Val Phe Phe Gln Arg Leu Gly Ile Ala Ser Gly Arg Ala Arg
465             470             475             480

Tyr Thr Lys Asn Trp Lys Thr Asn Lys Val Ser Ser Tyr Pro Leu Tyr
            485             490             495

His Ser Val Tyr Glu Thr Tyr Glu Leu Val Val Lys Phe Tyr Asp Pro
            500             505             510

Thr Phe Lys Tyr His Leu Thr Val Ala Gln Val Arg Gly Ala Met Val
            515             520             525

Phe Glu Leu Ala Asn Ser Ile Val Leu Pro Phe Asp Cys Gln Ser Tyr
        530             535             540

Ala Val Ala Leu Lys Lys Tyr Ala Asp Thr Ile Tyr Asn Ile Ser Met
545             550             555             560

Lys His Pro Gln Glu Met Lys Ala Tyr Met Ile Ser Phe Asp Ser Leu
            565             570             575

Phe Ser Ala Val Asn Asn Phe Thr Asp Val Ala Ser Lys Phe Asn Gln
            580             585             590

Arg Leu Gln Glu Leu Asp Lys Ser Asn Pro Ile Leu Leu Arg Ile Met
            595             600             605

Asn Asp Gln Leu Met Tyr Leu Glu Arg Ala Phe Ile Asp Pro Leu Gly
            610             615             620

Leu Pro Gly Arg Pro Phe Tyr Arg His Thr Ile Tyr Ala Pro Ser Ser
625             630             635             640

His Asn Lys Tyr Ala Gly Glu Ser Phe Pro Gly Ile Tyr Asp Ala Leu
            645             650             655

Phe Asp Ile Ser Ser Lys Val Asn Ala Ser Lys Ala Trp Asn Glu Val
            660             665             670
```

34

Lys Arg Gln Ile Ser Ile Ala Thr Phe Thr Val Gln Ala Ala Ala Glu
        675   680   685

Thr Leu Arg Glu Val Ala
        690

<210> 11
<211> 45
<212> DNA
<213> Clostridium tetani

<220>
<221> CDS
<222> (1)..(45)

<400> 11
cag tac atc aaa gct aac tcc aaa ttc atc ggt atc acc gag ctg   45
Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
  1   5   10   15

<210> 12
<211> 15
<212> PRT
<213> Clostridium tetani

<400> 12
Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
  1   5   10   15

<210> 13
<211> 63
<212> DNA
<213> Clostridium tetani

<220>
<221> CDS
<222> (1)..(63)

<400> 13
ttc aac aac ttc acc gta agc ttc tgg ctg cgt gtt ccg aaa gtt agc   48
Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
  1   5   10   15

gct agc cac ctg gaa   63
Ala Ser His Leu Glu
        20

```
<210> 14
<211> 21
<212> PRT
<213> Clostridium tetani

<400> 14
Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
  1               5                  10                  15

Ala Ser His Leu Glu
            20



<210> 15
<211> 25
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Fusion of
      tetanus toxoid epitope and PSM

<400> 15
Gln Glu Arg Gly Val Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly
  1               5                  10                  15

Ile Thr Glu Leu Arg Val Asp Cys Thr
            20                  25



<210> 16
<211> 25
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Fusion of
      tetanus toxoid epitope and PSM

<400> 16
Ala Val Val Leu Arg Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly
  1               5                  10                  15

Ile Thr Glu Leu Glu Met Lys Thr Tyr
            20                  25



<210> 17
<211> 25
<212> PRT
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Fusion of
      tetanus toxoid epitope and PSM

<400> 17
Met Phe Leu Glu Arg Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly
 1   5   10   15

Ile Thr Glu Leu His Val Ile Tyr Ala
            20   25

<210> 18
<211> 31
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Fusion of
      tetanus toxoid epitope and PSM

<400> 18
Asn Ser Arg Leu Leu Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg
 1   5   10   15

Val Pro Lys Val Ser Ala Ser His Leu Glu Val Asp Cys Thr Pro
            20   25   30

<210> 19
<211> 31
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Fusion of
      tetanus toxoid epitope and PSM

<400> 19
Val Val Leu Arg Lys Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg
 1   5   10   15

Val Pro Lys Val Ser Ala Ser His Leu Glu Ser Phe Asp Ser Leu
            20   25   30

<210> 20
<211> 31

```
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Fusion of
      tetanus toxoid epitope and PSM

<400> 20
Leu Met Phe Leu Glu Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg
 1               5                  10                  15

Val Pro Lys Val Ser Ala Ser His Leu Glu Pro Ser Ser His Asn
            20                  25                  30



<210> 21
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Artificial His
      tag

<220>
<221> CDS
<222> (1)..(18)

<400> 21
cat cat cat cat cat cat                                           18
His His His His His His
 1               5



<210> 22
<211> 6
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Artificial His
      tag

<400> 22
His His His His His His
 1               5



<210> 23
<211> 42
<212> DNA
<213> Artificial Sequence
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38

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<220>
<223> Description of Artificial Sequence: Artificial His
      tag

<220>
<221> CDS
<222> (1)..(42)

<400> 23
atg aaa cac caa cac caa cat caa cat caa cat caa cat caa        42
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln
  1               5                  10


<210> 24
<211> 14
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Artificial His
      tag

<400> 24
Met Lys His Gln His Gln His Gln His Gln His Gln His Gln
  1               5                  10



<210> 25
<211> 69
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(69)

<400> 25
atg gat gca atg aag aga ggg ctc tgc tgt gtg ctg ctg ctg tgt gga    48
Met Asp Ala Met Lys Arg Gly Leu Cys Cys Val Leu Leu Leu Cys Gly
  1               5                  10                  15

gca gtc ttc gtt tcg ccc agc                                     69
Ala Val Phe Val Ser Pro Ser
                20


<210> 26
<211> 23
<212> PRT
<213> Mus musculus

<400> 26
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39

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Met Asp Ala Met Lys Arg Gly Leu Cys Cys Val Leu Leu Leu Cys Gly
 1               5                   10                  15

Ala Val Phe Val Ser Pro Ser
            20




<210> 27
<211> 33
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(33)

<400> 27
gaa caa aaa ctc atc tca gaa gag gat ctg aat                    33
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn
 1               5                   10




<210> 28
<211> 11
<212> PRT
<213> Homo sapiens

<400> 28
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn
 1               5                   10




<210> 29
<211> 75
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(75)

<400> 29
atg aag gat tcc tgc atc act gtg atg gcc atg gcg ctg ctg tct ggg    48
Met Lys Asp Ser Cys Ile Thr Val Met Ala Met Ala Leu Leu Ser Gly
 1               5                   10                  15

ttc ttt ttc ttc gcg ccg gcc tcg agc                            75
Phe Phe Phe Phe Ala Pro Ala Ser Ser
            20                  25
```

```
<210> 30
<211> 25
<212> PRT
<213> Homo sapiens

<400> 30
Met Lys Asp Ser Cys Ile Thr Val Met Ala Met Ala Leu Leu Ser Gly
 1               5                  10                  15

Phe Phe Phe Phe Ala Pro Ala Ser Ser
             20                  25
```

```
<210> 31
<211> 60
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(60)

<400> 31
atg aga agg atg ctt ctg cac ttg agt gtt ctg act ctc agc tgt gtc    48
Met Arg Arg Met Leu Leu His Leu Ser Val Leu Thr Leu Ser Cys Val
 1               5                  10                  15

tgg gcc act gcc                                                    60
Trp Ala Thr Ala
             20
```

```
<210> 32
<211> 20
<212> PRT
<213> Mus musculus

<400> 32
Met Arg Arg Met Leu Leu His Leu Ser Val Leu Thr Leu Ser Cys Val
 1               5                  10                  15

Trp Ala Thr Ala
             20
```

```
<210> 33
<211> 20
<212> PRT
<213> Homo sapiens

<400> 33
```

Pro Ala Pro Gly Ser Thr Ala Pro Pro Ala His Gly Val Thr Ser Ala
 1   5   10   15

Pro Asp Thr Arg
            20